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It is characteristic of Science and Progress that they continually open new fields to our vision—PASTEUR

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ON THE "ACTIVATION" OF THE LACTASE OF ESCHERICHIA COLI-MUTABILE[1]

CHARLES J. DEERE

Department of Chemistry, University of Tennessee School of Biological Sciences, Memphis

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The studies reported in this paper were directed to an explanation of the puzzling phenomenon that the non-lactose-fermenting *Escherichia coli-mutabile* contains lactase (Deere, Dulaney and Michelson, 1939). Earlier experiments led us to believe that the antiseptics employed "activated" the lactase which was present, but inactive, in living growing cultures of the non-lactose-fermenting (white) form. Use of an antiseptic was at first necessary in determinations of lactase activity since it protected the digest against contaminants and rendered the preparations incapable of attacking glucose and galactose. It was later found that lactase activity of preparations of the white form did not depend on the presence of, or preliminary treatment with, an antiseptic. Drying of cell suspensions by vacuum distillation suffices to "activate" the lactase.

We have investigated more extensively the effect of drying on the various enzyme activities of these organisms. The drying process, unlike the various lactase "activating" antiseptics which we have employed, only partially inhibits the enzyme system or systems concerned in the metabolism of glucose and galactose. Hence, reduction methods for sugars present great difficulties in evaluating results obtained on digests in which the reducing value is being increased by one process (lactose hydrolysis) and

[1] The data in this paper are taken from a dissertation presented by Charles J. Deere to the Committee on Graduate Study in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Tennessee, September, 1937.

decreased by another (monose metabolism). Since a portion of the sugar metabolism of *Escherichia coli-mutabile* involves the consumption of oxygen, this oxidative phase apparently being limited to monose metabolism, we have attempted herein to analyze enzyme activities from measurements of oxygen consumption. For brevity we shall designate the enzyme system responsible for oxygen consumption as the "oxidase system." The over-all increase in oxygen consumption by dried organisms in lactose, as contrasted with non-dried organisms in lactose, is to be attributed to a primary hydrolysis of the lactose in the first instance and an only partially destructive effect of the drying process upon the subsequent monose metabolism.

METHODS

Oxygen consumption

Oxygen consumption of cell suspensions was measured in a Warburg apparatus at 37.5°C. Carbon dioxide was absorbed by a roll of filter paper moistened with 0.1 cc. of 20 per cent potassium hydroxide.

Cell preparations

The cells were grown on plain or sugar agar and cell suspensions were prepared as described previously (Deere, Dulaney and Michelson, 1939). One-cubic centimeter quantities of cell suspensions were dried in weighed Warburg flasks by vacuum distillation. The weight of dried cells was determined and 1 cc. of distilled water added to replace that lost in drying. One-cubic centimeter portions of cell suspensions which had not been dried served as controls.

Procedure

To the aqueous suspensions of dried or non-dried cells in the Warburg flasks were added 1 cc. of 1.5 per cent sodium chloride in 0.3 M phosphate buffer (pH 7.0) and 1 cc. of 1.5 per cent sugar solution or (for control) 1 cc. of distilled water. The flasks were attached to manometers and placed in the bath. Fifteen minutes later the vessels were shut off from the air and the pressure

changes recorded at 15-minute intervals. With samples whose oxygen consumption was high, the vessels had to be opened at each 15-minute interval. When this was necessary, 1 minute elapsed between successive intervals.

RESULTS

In experiment 1 (table 1) is reported the oxygen consumption of suspensions of 24-hour plain-agar cultures of Garrett white. In lactose, the dried sample (sample 4) consumed 2.5 times as much oxygen as the non-dried sample (sample 10). The oxygen consumption of dried cells (sample 8) in glucose was about two-thirds that of non-dried cells in glucose (sample 18). These results demonstrate a partial inhibition of the oxidase system by drying but an "activating" effect on lactase. The oxygen consumption of dried cells suspended in glucose is about 3 times that of similarly treated cells suspended in lactose. This finding is not surprising in view of our previous demonstration (Deere, Dulaney and Michelson, 1939) that cells grown in the absence of lactose have only slight lactase activity.

A similar experiment (experiment 2, table 1) was performed using suspensions of Garrett white which had been grown for 24 hours on 1 per cent lactose agar. The results are qualitatively no different from the results reported in experiment 1. Dried cells (sample 8) consumed slightly less oxygen in glucose than non-dried cells (sample 18) but, in lactose, dried cells (sample 4) consumed 3.7 times as much oxygen as non-dried cells (sample 10). Non-dried cells (sample 3) consumed about one-half as much oxygen in the absence of sugar as similarly treated cells suspended in lactose (sample 10). These results demonstrate again the "activating" effect of drying on the lactase of this organism. Comparison of these results with experiment 1 demonstrates the large increase in lactase content which occurs when this organism is grown on lactose.

We then turned our attention to the behavior of the lactose-fermenting (red) strain in similar experiments. Experiment 3 (table 1) gives the results obtained with a suspension of a 24-hour growth of Garrett red on 1 per cent lactose agar. It will be noted

TABLE 1

Oxygen consumption of suspensions of Escherichia coli-mutabile

Experiment Number.	1				2					3				4		5			6		
Strain used	White				White					Red				Red		White			Red		
Culture medium	Plain				Lactose					Lactose				Plain		Glucose			Plain		
Sample number	10	4	18	8	3	10	4	18	8	18	4	8	10	16	18	7	16	18	7	16	10
Treatment of cells	Non-dried	Dried	Non-dried	Dried	Non-dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Non-dried	Dried	Dried	Non-dried
Weight of sample, mgm.	—	9.3	—	9.2	—	—	9.1	—	8.9	—	9.1	9.0	—	10.0	—	7.9	—	—	8.9	8.4	—
Sugar present in respirometer	Lactose	Lactose	Glucose	Glucose	None	Lactose	Lactose	Glucose	Glucose	None	Lactose	Glucose	Lactose	Glucose	Glucose	Glucose	Glucose	None	Glucose	Lactose	Lactose
Time	Oxygen consumption in cubic millimeters per 10 mgm. of preparation																				
minutes																					
0–15	16	46	164	126	7	19	89	230	212	7	10	0	120	89	175	−3	199	16	112	46	27
15–30	13	41	183	125	19	23	87	196	189	8	3	1	148	86	170	1	205	19	108	42	7
30–45	17	42	196	117	7	22	83	201	196	12	4	6	166	80	167	5	193	16	116	52	30
45–60	15	33	183	119	7	19	90	175	167	13	0	3	165	88	161	8	199	19	112	50	16
60–75	11	35	167	102	10	26	95	161	157	10	2	1	173	79	154	8	185	14	117	56	29
75–90	16	33	172	110	13	28	91	160	157	9	−3	0	173	69	87	5	167	13	100	55	19
90–105	14	38	168	116	14	25	95	132	134	9	−1	1	168	78	139				121	62	36
105–120	15	39	156	102	12	28	96	103	107	7	4	6	166	76	118				103	60	28
Total oxygen consumption	117	307	1,389	917	89	190	726	1,358	1,319	75	19	18	1,279	645	1,171				889	423	192

that only a slight oxygen consumption by dried preparations occurs in lactose or glucose (samples 4 and 8). These results indicated that destruction of the oxidase system had occurred during the drying procedure, earlier work (Deere, Dulaney and Michelson, 1939) having demonstrated that lactase is not damaged by drying. Drying had not destroyed the oxidase system of the white strain (experiments 1 and 2). The most plausible explanation of the difference in behavior of the two strains appeared to be the difference in pH of the medium from which the

TABLE 2

Oxygen consumption of suspensions of Garrett red grown on lactose agar

Samples 10, 16 and 18 washed in $NaHCO_3$ to bring pH to 7.6

Substrate—0.1 M phosphate, pH 7.0, 0.5 per cent sodium chloride, and 0.5 per cent sugar

Sample Number...	4	7	8	10	16	18
Treatment of cells..	Dried	Dried	Non-dried	Dried	Dried	Non-dried
Weight of sample, mgm.....	5.6	5.6	—	4.7	4.4	—
Sugar present.....	Lactose	Glucose	Glucose	Lactose	Glucose	Glucose
Time interval	Oxygen consumption in cubic millimeters					
minutes						
0–15	2	1	147	1	4	138
15–30	2	0	124	0	2	123
30–45	0	2	111	−2	4	117
45–60	1	1	101	1	−1	96
Total oxygen consumption	5	4	483	0	9	474

cells had been harvested. 24-hour growth on lactose agar produced a pH of about 5 for the red and above 7 for the white strain. It appeared unlikely that more than traces of organic acids would remain in the cells of the red strain during the process of washing, once in saline and twice in distilled water. This possibility, however, was now tested.

A 26-hour growth of Garrett red on 1 per cent lactose agar was harvested in saline as usual and the resulting suspension divided into two parts. One part was centrifuged without further

treatment. The second part was treated with an equal volume of M/10 sodium bicarbonate and then centrifuged. The supernatant fluid from the bicarbonate-treated sample had a pH of 7.6; the untreated sample had a pH of 4.9. Both samples were washed twice in distilled water in the usual manner and portions prepared for study of the effect of drying on oxygen consumption. The results (table 2) demonstrate a similar behavior of the non-treated and bicarbonate-treated samples. The non-dried samples consumed oxygen rapidly in glucose but the dried samples consumed negligible quantities of oxygen in lactose or glucose. Thus, it appeared that the oxidase system of the red strain was peculiarly susceptible to destruction by drying or that growth on a fermentable sugar produced a susceptibility to destruction, a susceptibility which was not altered by neutralization of the products of fermentation before drying.

We therefore tested the effect of drying on the oxidase activity of a plain agar culture of the red strain. A 23-hour growth of Garrett red gave a pH of 7.3, a reaction similar to that of a lactose agar culture of the white strain. The results (experiment 4, table 1) demonstrate the failure of the drying technique to paralyze the oxidase system of cells of the red strain grown in this manner. It appears then that growth of the red strain on a fermentable sugar is responsible for the susceptibility of its oxidase system to destruction by drying. It seemed unlikely that this behavior was peculiar or characteristic of only the red strain. This indicated the advisability of studying the behavior of suspensions of the white strain which had been grown on a fermentable sugar.

A 24-hour culture of Garrett white on 1 per cent glucose agar was treated in the usual manner and the effect of drying on the oxygen consumption of cells suspended in glucose was determined. The destructive effect of drying on the oxidase system of these cells is apparent (experiment 5, table 1). Our original plan of comparing the effect of drying on the lactase activity of the two strains could then be carried out. This required a dry preparation which retains oxidase activity which could be obtained with the red strain only by growing it on plain agar.

Study of a 25½-hour plain-agar culture of Garrett red revealed that drying doubled the oxygen consumption of cells suspended in lactose (experiment 6, table 1). Comparison of these results with those of experiment 1 and 2 demonstrates a similarity in the activating effect of drying on the lactase activity of both the white and red strains though the activation is more marked with the white.

It occurred to us that the increase in lactase activity which takes place on drying might be due to release of lactase from cells into the surrounding fluid. An experiment was designed to test this hypothesis. A 25-hour growth of Garrett white was harvested from 1 per cent lactose agar, the pH being 7.2. The cells were washed in the usual manner and resuspended in water. Four 1-cc. portions of the suspension were measured into Warburg flasks and set aside for respiration studies, after one of them, no. 7, had been dried by vacuum distillation. The weight of dry material was 7.1 mgm. Two 3 cc. portions of the suspension were measured into centrifuge tubes and used for preparation of extracts A and B.

The sample used for preparation of extract A was dried in a 50 cc. centrifuge tube by vacuum distillation, the weight of dry material being 21.3 mgm. After the dried sample had stood for 2½ hours, 3 cc. of 1.5 per cent sodium chloride in 0.3 M phosphate buffer were added to it. The cells were resuspended and 15 minutes later the sample was centrifuged. One cubic centimeter of the supernatant fluid represents the extract from 7.1 mgm. of dried cells and is designated as extract A.

A second 3-cc. portion of the original suspension was centrifuged and the supernatant fluid removed as completely as possible. Then 3 cc. of the phosphate-sodium chloride solution were added, the cells resuspended, and 5 minutes later the sample was centrifuged. One cubic centimeter of the supernatant fluid represents the extract from 7.1 mgm. of non-dried cells and is designated extract B.

For determination of the effect of these extracts on the oxygen consumption of cells, samples containing cells alone, extract alone, and cells in the presence of extract were prepared. Lac-

tose was added to all samples. The results of the experiment are presented in table 3.

Extract B (of non-dried cells) consumes no oxygen in the presence of lactose (sample 16). Addition of extract B to non-dried cells suspended in lactose (sample 8) results in no greater oxygen consumption than was observed in the absence of extract (sample 10). These facts are interpreted as indicating the absence of lactase in extract B.

TABLE 3

The lactase activity of an extract of dried cells of Garrett white

Substrate—0.1 M phosphate, pH 7.0, 0.5 per cent sodium chloride, and 0.5 per cent lactose

Extract A is an extract of dried cells. Extract B is an extract of non-dried cells

Sample Number...	8	16	10	4	7	18
Type of cells present.....	Non-dried	None	Non-dried	Non-dried	Dried	None
Extract present....	B	B	None	A	None	A
Time interval	Oxygen consumption in cubic millimeters					
minutes						
0–15	6	−2	4	19	62	−3
15–30	11	2	14	17	62	−7
30–45	8	−1	5	18	65	0
45–60	11	0	12	17	64	−1
60–75	10	0	10	18	56	−2
75–90	11	0	10	18	56	0
Total oxygen consumption	57	−1	55	107	365	−13

Extract A (of dried cells) produces gas when added to lactose (sample 18). Since potassium hydroxide was present to absorb carbon dioxide, it seems probable that some hydrogen was produced. Addition of extract A to non-dried cells suspended in lactose (sample 4) results in an oxygen consumption 1.9 times that observed in the absence of extract (sample 10). We attribute these results to the presence of lactase in extract A.

DISCUSSION

The results reported here confirm previously reported findings that the difference in the two strains of *Escherichia coli-mutabile*

does not lie so much in lactase content as in lactase activity, the lactase of the white strain being inactive in uninjured cells. Several possible explanations of the presence of an inactive enzyme in a cell can be offered. One explanation is the presence of a specific enzyme inhibitor or antienzyme as postulated by Stewart (1926 and 1928). Our results do not contradict this hypothesis. In fact, our results could be explained easily if it could be shown that an antienzyme is, in reality, present. However, if this explanation is correct, the antienzyme is very labile for it is destroyed by antiseptics or by drying. We have considered trying to extract such a substance but, if it is present, its extreme lability has made the possibility of extracting it exceedingly remote.

Stewart believed that the variants were formed through the loss of this hypothetical antienzyme by growth on lactose. He assumed, but did not prove, the presence of lactase in the white form of *Escherichia coli-mutabile*. Lewis (1934) does not accept Stewart's view. Lewis believes he has demonstrated that the mutation of *Escherichia coli-mutabile* occurs continuously, irrespective of the medium.

A possible explanation which seems plausible to us is based on permeability of the bacterial cell. Is it not possible that the failure of the white strain to metabolize lactose is due to impermeability of the cell to lactose rather than to inactivity (antienzyme suppression) of its lactase? Search of the literature has revealed no answer to the question of the permeability of bacterial cell membranes to sugars. Lactase of this group of organisms is generally agreed to be intracellular. The methods which we have used for demonstration of lactase in *Escherichia coli-mutabile* undoubtedly injure the cells. Since uninjured cells of the white strain do not metabolize lactose, and since injury which is known to influence permeability renders them active towards lactose, it seems reasonable to suggest that the inability of the uninjured cells to metabolize lactose is due to their impermeability to lactose. We have demonstrated that significant amounts of lactase can be extracted from injured (dried) cells (table 3) but change in permeability of the cell to lactose may be of more consequence than change in permeability to lactase.

An hypothesis based upon permeability of the cells must explain the following facts:

1. The white strain contains lactase but is unable to ferment lactose. Impermeability of the cells to lactose would satisfactorily explain this fact.

2. Prolonged growth of the white strain in the presence of lactose results in the production of lactose-fermenting variants (reds). This transformation could be explained as a change from a cell impermeable to lactose to one permeable to it.

3. Treatment of cells of the white strain with antiseptics or simply drying them renders their lactase active. These treatments are injurious to the cell and cell injury is known to alter permeability. These facts fit well in the hypothesis.

4. Drying cells of the red strain increases their lactase activity as judged by their oxygen consumption in the presence of lactose. This fact can be reconciled with the permeability hypothesis on the assumption that the uninjured cells are difficultly permeable to lactose and that the drying process increases this permeability.

SUMMARY

Studies on the oxygen consumption of preparations of *Escherichia coli-mutabile* have demonstrated the following facts:

1. Lactase activity of preparations of the white strain does not depend on the presence of or preliminary treatment with an antiseptic. High vacuum drying suffices to increase the oxygen consumption in lactose to 3 to 6 times that observed with non-dried preparations.

2. Our previous demonstration of the necessity of the presence of lactose in the medium on which cells are grown in order to obtain high lactase activity is confirmed by the present study.

3. High-vacuum-dried preparations of the red strain harvested from lactose agar consume no oxygen when suspended in lactose. This behavior was shown to be due to inactivation of the oxidase system by the drying procedure.

4. Drying cells of the white strain which have been grown on glucose results in inactivation of their oxidase system. This phenomenon was observed with both the white and red strains

when they had been grown on fermentable carbohydrate and was not observed when they were grown in the absence of fermentable carbohydrate. The significance of this observation is not apparent.

5. Drying cells of the red strain which have been grown on plain agar yields a preparation which consumes more oxygen when suspended in lactose than does the corresponding non-dried preparation. In this case the increase in oxygen consumption is not as marked as with the white strain.

6. Extracts of dried cells of the white strain increase the oxygen consumption of non-dried cells suspended in lactose. A similar extract of non-dried cells does not have this effect.

An hypothesis of altered membrane permeability which attempts to explain the observed facts is presented.

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THE INTRODUCTION OF AGAR-AGAR INTO BACTERIOLOGY

ARTHUR PARKER HITCHENS

Army Medical School, Washington, D. C.

AND

MORRIS C. LEIKIND

Institute of the History of Medicine, Johns Hopkins University, Baltimore, Md.

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Among fundamental developments in bacteriology the elaboration of solid culture substrates will always hold first place. With the introduction of a method for isolating microörganisms and growing them in pure culture, bacteriologists possessed the means for studying what Ferdinand Cohn (1872) called "Die kleinsten lebenden Wesen." Credit for the discovery of the solid medium technique is commonly accorded to Robert Koch, but, as is usual with discoveries of major importance in science, he had his fore-runners. Of course this detracts in no way from the glory of Koch's achievement; his genius lay in his ability to bring order out of chaos. Starting as it were with a box of miscellaneous beads, varying in size and shape, each bead a scientific fact, he found a thread on which the beads could be strung to form a perfect necklace.

There are, it seems, two types of workers in Science, the bead collectors and the bead stringers. Which of the two is more important must not be a question for discussion here. It is a fact, however, that bead collectors are more numerous than bead stringers and it is also an historical commonplace that the bead collectors are more obscure, less known to the world, than those geniuses who string necklaces. The historian of science must concern himself with both types of workers and their productions. The contributions of the obscure are sometimes as important as those of investigators in a more favorable position to be il-

luminated by the spotlight of fame. Yet history does record the work of individuals, who, while generally unknown and unsung have made discoveries of lasting value. As a foreword or "Golden Text" to his volume *Behind the Doctor*, Clendening (1933) quotes from Oliver Wendell Holmes that medicine learned "from a Jesuit how to cure agues, from a friar how to cut for the stone, from a soldier how to treat gout, from a sailor how to keep off scurvy, from a postmaster how to sound the Eustachian tube, from a dairymaid how to prevent smallpox, and from an old market-woman how to catch the itch-insect."

To this list we can now add a housewife and tell the story of how she helped her bacteriologist husband in his studies upon the microörganisms of the air and thereby achieved pure cultures. For many years it has been known to bacteriologists that agar-agar was introduced into bacteriological technique by a certain Frau Hesse. But who Frau Hesse[1] was and how she came upon this important innovation has been until now generally unknown. In August of 1937 we came into the possession of additional facts concerning this discovery. The story of Fannie Eilshemius Hesse is, we feel, of interest to all bacteriologists, but especially to American students of this branch of science. Following the publication of his brief memorial notice in the Zentr. f. Bakt., 1935, we wrote to Professor Edgar von Gierke (1935) asking if he would put us on the track of information we had been seeking for many years. He kindly referred our letter to Dr. Friedrich Hesse, a surgeon now living in Dresden, Germany. He is the son of Frau Hesse and graciously provided us with the facts upon which our story is based. We have also received from him portraits of his parents, here reproduced for the first time. For these facts and photographs, bacteriologists in general owe a debt of gratitude to Dr. Friedrich Hesse.

Frau Hesse was born Fannie Eilshemius in 1850 in a locality now incorporated in Jersey City in the state of New Jersey.

[1] There are two brothers of Frau Hesse still living in New York City, Mr. Henry and Mr. Louis Eilshemius. The latter is a well known artist, his work being the subject of an illustrated paper published in the October (1937) number of the *Magazine of Art*. He has often abbreviated his name to Elshemus.

At that time this community was known as Laurel Hill. Her father had immigrated here from Germany in 1818. We know little of her early life. In 1874 she went abroad, traveling over

FRAU FANNY EILSHEMIUS AND DR. WALTHER HESSE

Europe and finally coming to Germany. It was there she met Walther Hesse, then a district physician in Schwartzenberg, Saxony. Young Hesse was more than a mere country doctor and health officer. He was aware of the new scientific horizons

appearing in the work of Pasteur, Koch, Ferdinand Cohn and many others, for in the winter of 1881–82 he became a student of the then new science of Bacteriology under Robert Koch in the laboratories of the *Kaiserliche Gesundheitsamt*, in Berlin. Many problems were pressing for solution in those early days and although the most important were those dealing with the bacteria causing infectious disease, the problem of the general distribution of these organisms was not neglected. Hesse (1884) began to study the bacterial content of the air.[2] These studies were continued at his home in Schwartzenberg after his half-year period of residency in the Imperial Health Office. It was in his home, now also his laboratory, that the momentous discovery was made. To understand the importance of this new technical achievement we must review briefly a few facts regarding the historical development of pure culture methods.

As we now know so well from the work of Dobell (1932) and the translation of Cohen (1937), bacteria had been seen and described by Leeuwenhoek just a little over two hundred years before Hesse began his research. During most of the intervening period observers had been content to study them as they were found in nature. Until the time when Pasteur (1857) began his researches on fermentation few individuals had seemed to be interested in whether bacteria could be grown or not. Except to a few botanists, especially Ferdinand Cohn (1872), the problem of the classification of species and genera was of little moment. Most students agreed with Linnaeus (1763) that bacteria belonged in the class *Chaos;* they were little advanced beyond the early classification made by Otho Fridericus Müller (1773). With the studies of Pasteur (1857), Cohn (1872), and Koch (1881, 1882) a new era began. Pasteur, although no systematist, demonstrated the physiological specificity of bacteria. In his studies on fermentation he showed that each type of the organisms which he called by so many different

[2] The results of this work were finally published in vol. II (1884) of the Contributions from the Gesundheitsamt. The paper is illustrated by three double page chromolithographs. The original paintings for these were made by Frau Hesse. They are in every way excellent.

names was characterized by and responsible for specific physiological activities. In order to prove his contention that specific organisms could bring about alcoholic, lactic or butyric fermentations he devised simple nutrient fluids of known composition to demonstrate these reactions. By transferring minute quantities of fluid containing bacteria from flask to flask he was able to obtain relatively pure cultures. At least they were pure on the basis of the fermentations brought about. There were various attempts to modify his medium and many workers, among them Klebs (1873), Lister (1878), and Salomonsen (1876), attacked the problem of growing pure cultures, with varying degrees of success. The great stumbling block was that they were all working with fluid culture media and the difficulties were almost insuperable. But another group of workers, especially those interested in fungi, were attacking the problem from a more productive angle. As related so dramatically by Harrison (1924) and echoed by Reid (1936) people had for centuries been terrified and awed by epidemics of "blood spots" miraculously appearing on food stuffs, especially the holy wafers. In 1819 an extensive and persistent epidemic of "bloody bread" broke out in Padua. An official investigation was made and one of the investigators, Bartolomeo Bizio, recognized the fungus nature of the blood-red growth. He isolated it from some corn porridge (polenta) and carried out a series of successful transfers to other farinaceous materials. Bizio (1823) named the organism *Serratia marcescens: Serratia*, in honor of Serafino Serrati, who was the first to run a steamboat on the Arno, the species name signifying putrefaction. Bizio is not heard of further but we may date the first attempts to grow bacteria on solid culture media from his experiments. Observations were made concerning the more or less accidental growth of bacteria on various organic solid media in the years that followed, but there were none planned with definite and serious purpose until we come to the work of Schroeter (1872), a pupil of Ferdinand Cohn. The paper that he published in 1872 on the chromogenic bacteria is a landmark. He was able to separate various chromogens from each other by growing them on solid media such as potato, coagu-

lated egg white, starch paste and meat. Schroeter found that on these solid substrates his pigment producers often appeared as isolated spots of color. He then learned that all organisms in a single spot or colony were alike. When he transferred organisms from a single colony to fresh media he was able to grow the same organisms and, by repeated transfer, to continue the process indefinitely. He had there the secret of pure culture study but technical difficulties stood in the way of the universal application of his method. The difficulties were due to the presence of nonchromogenic bacteria, especially when the unpigmented colonies closely resembled the color of the medium. It was only a few years later that Koch (1881) entered the field and his genius promptly solved these disturbing problems. He recognized that one must have a substrate which was at once solid, transparent and sterile. What he sought was a universal culture medium, but he soon recognized the impracticability of this quest. He then turned his attention to finding a suitable jelly that could be incorporated with a nutrient fluid. His most promising material was gelatin which the mycologists, among them Vittadini (1850), had been using, for 30 years. With the aid of this medium and his plating and dilution method, Koch (1881) revolutionized bacteriological technique. Isolating pure cultures was, in comparison with the older techniques, enormously simplified. However, the way was not yet completely cleared. Gelatin was not quite the ideal jellifying medium required. It possessed two undesirable qualities. First, it was liquefied by certain organisms. This characteristic, while of value in the identification of a lytic ferment, ruined the medium for the purposes of isolating pure cultures. The second defect arose from the fact that gelatin is not solid but fluid at 37°C. incubator temperature. It was satisfactory for organisms cultivable at room temperatures—22°C., or below—but not for pathogens requiring blood heat.

It was with these difficulties that Walther Hesse and his wife, Fannie, wrestled. Fannie was not merely the "Hausfrau," she was also the technician and the artist illustrator in the home

and laboratory of Dr. Hesse. She cooked not only the soup for her family but also bouillon for her husband's bacteria. In the studies of atmospheric bacteria made by Dr. Hesse (1884), tubes lined with gelatin were used. Measured quantities of air were aspirated through these tubes and from the colonies of bacteria developing on the lining medium the numbers and kinds of microbes in the air were listed. But the maddening liquefaction of gelatin ruined many of the experiments and finally Hesse began to seek new solidifying agents. At this point Frau Hesse became an historic figure. She suggested the use of agar-agar which she had been using for years in her kitchen in the preparation of fruit and vegetable jellies. While yet in America she had received the recipe from her mother; her mother in turn had obtained the formula from some Dutch friends who had formerly lived in Java. In the East Indies, where the source of agar-agar, Japanese seaweed (*Gelidium corneum*), also abounds, this curious material had been used for generations as a jellifying agent and as a thickening for soups (Smith, H. M., 1905).

We can imagine the elation of Dr. Hesse when he set up and studied his tubes lined with his wife's new medium and found that his troubles were at an end. Now he could prepare a substrate, solid, transparent and sterile, which would retain its consistency at all temperatures at which bacteria could grow and which, furthermore, would not be liquified by any of the organisms he encountered in his studies. Without delay the discovery was communicated to Robert Koch by letter, probably late in 1881. Koch recognized its value and made it his own. In 1882, in his now classic preliminary note on the tubercle bacillus, Koch made what is the first printed reference to the use of agar—just one short sentence for a technical improvement so fundamental and epoch making. No formal paper was ever published.

Thus did a modest housewife perform a service to science and to humanity. When she died in 1934, few bacteriologists knew of her death, few perhaps that she had ever lived. Lesser innovations and discoveries are commemorated with the name of

the discoverer. Could not "plain agar" from now on be designated as "Frau Hesse's medium?" Her contribution to bacteriology makes her immortal.

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NICOTINIC ACID AND THIAMIN HYDROCHLORIDE AS GROWTH-PROMOTING FACTORS FOR BRUCELLA

GRACE P. KERBY

From the Brucellosis Laboratory, Clayton Foundation for Research, Nix Professional Building, San Antonio, Texas

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Nicotinic acid and vitamin B-1 (thiamin hydrochloride) have aroused considerable interest as growth-promoting substances for bacteria in artificial media. Mueller (1937), working with the diphtheria bacillus, found that nicotinic acid accounts for a part of the growth-promoting activity of tissue extracts for that organism, its maximal effect being exerted "at a concentration of about 1 mgm. per liter of medium but varying somewhat with the composition of the control medium." Knight (1937, a and b) demonstrated the necessity of nicotinic acid and its amide for the growth of *Staphylococcus aureus*, his findings being confirmed later by Landy (1938). Knight (1937 c) also found that, in addition to nicotinic acid, vitamin B-1 is required by the staphylococcus. In the present study an attempt has been made to determine (1) if these substances, when added to routine media, will enhance the growth of *Brucella* and (2) if so, what concentrations will produce maximal results.

Bacto Tryptose Agar has been used as the basic medium. Two recently isolated strains of *Brucella abortus* (*B. abortus* RV-3 and 1033), 4 stock strains of *B. abortus* (*B. abortus* G-1153, 456, 161 and 230) and one stock strain of *Brucella melitensis* were employed in the study. A uniform inoculum of 0.05 cc. of a suitably diluted broth culture was used throughout for each plate.

Nicotinic acid was added to the medium in amounts ranging from 4 to 40 mgm. per liter, and thiamin hydrochloride in a range of from 0.01 to 100 mgm. per liter. Studies of the two

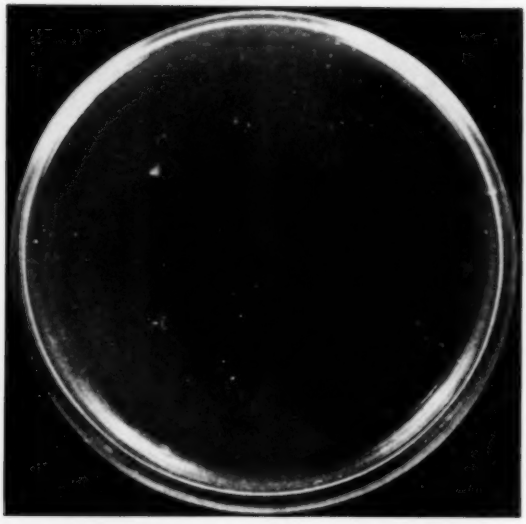

FIG. 1. GROWTH OF BRUCELLA ABORTUS 456 ON BACTO TRYPTOSE AGAR CONTROL

Plates were incubated 48 hours at 37°C. and 18 hours at room temperature before photographing.

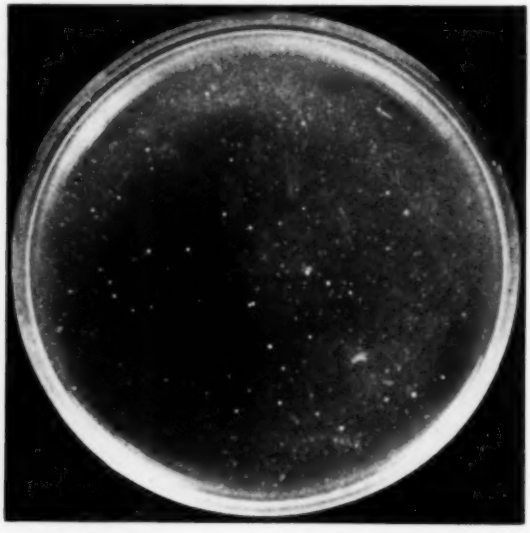

FIG. 2. GROWTH OF BRUCELLA ABORTUS 456 ON BACTO TRYPTOSE AGAR CONTAINING 30 MGM. NICOTINIC ACID PER LITER OF MEDIUM

Plates were incubated 48 hours at 37°C. and 18 hours at room temperature before photographing.

FIG. 3. GROWTH OF BRUCELLA ABORTUS 456 ON BACTO TRYPTOSE AGAR CONTAINING 25 MGM. THIAMIN HYDROCHLORIDE PER LITER OF MEDIUM

Plates were incubated 48 hours at 37°C. and 18 hours at room temperature before photographing.

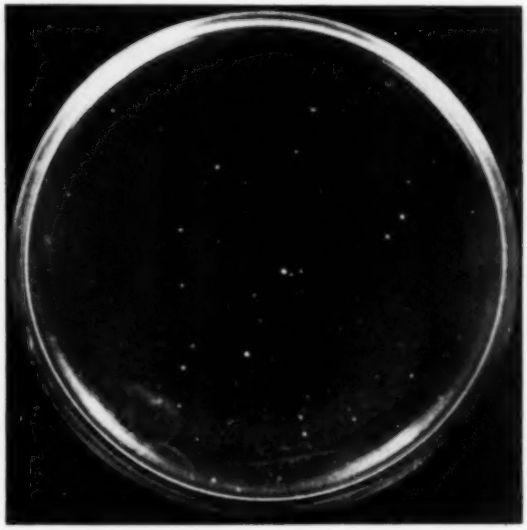

FIG. 4. GROWTH OF BRUCELLA ABORTUS 456 ON BACTO TRYPTOSE AGAR CONTAINING 30 MGM. NICOTINIC ACID AND 25 MGM. THIAMIN HYDROCHLORIDE PER LITER OF MEDIUM

Plates were incubated 48 hours at 37°C. and 18 hours at room temperature before photographing.

substances separately indicated that the optimal concentration of nicotinic acid was 30 mgm. per liter of medium and of thamin hydrochloride, 25 mgm. per liter.

Growth of all strains of *B. abortus* was enhanced, but to different degrees, by either nicotinic acid or thiamin hydrochloride. Thus, stock strains of *B. abortus* 456 and 161 grew better on the nicotinic acid medium, while stock strain *B. abortus* 230 grew better in the vitamin B-1 medium. Two recently isolated strains of *B. abortus* and stock strain *B. abortus* G-1153 grew best on the combination of nicotinic acid and thiamin hydrochloride. All strains of *B. abortus* showed as good or better growth on media containing both substances as they did when the materials were used separately.

The growth-promoting effect of these substances was noted mainly in the speed of initial growth; on the enriched media, colonies could be counted fully 24 hours before those on control plates. Above and below the optimal concentrations mentioned, the effect was diminished or lost. Ultimate size of colonies was also somewhat greater; but increase in number of colonies, while frequently observed, could not be considered significant because of the technical impossibility of insuring strictly uniform inocula.

In contrast to the effect of nicotinic acid and thiamin hydrochloride on the growth of *B. abortus*, the single stock strain of *B. melitensis* which was studied was inhibited slightly by the addition of either material. Whether this effect is typical of all melitensis strains must await further study.

Strains of *Brucella suis* were not investigated.

SUMMARY

The growth of *Brucella abortus* is enhanced by the addition of nicotinic acid and thiamin hydrochloride to Bacto Tryptose Agar in concentrations of 30 and 25 mgm., respectively, per liter of medium. Growth of certain strains is enhanced more by one than the other of these substances, but all strains grow well when both materials are added. For routine use, the combination seems advisable.

The single stock strain of *Brucella melitensis* studied was inhibited somewhat by either factor. *Brucella suis* was not investigated.

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THE OXIDATION OF ASCORBIC ACID AS INFLUENCED BY INTESTINAL BACTERIA[1,2]

WILLIAM B. ESSELEN, JR. AND JAMES E. FULLER

Department of Bacteriology and Physiology, Massachusetts State College, Amherst, Mass.

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INTRODUCTION

Stepp and Schroder (1935), and Stepp (1936), reported that certain strains of intestinal bacteria, particularly "*B. coli communis*" and "*B. paratyphosus B*", were capable of destroying ascorbic acid (vitamin C), while other intestinal bacteria were without action on this substance. They discussed the possibility that some cases of scurvy might be caused by the destruction of ascorbic acid by bacteria in the upper intestinal tract before the acid could be absorbed. Marin (1936) described a clinical case of scurvy which he believed had been caused by an "infection of *B. coli* and *B. paratyphosus B* in the upper intestinal tract."

Prompted by the above work the investigation here reported was carried on to study the action of normal intestinal bacteria on ascorbic acid, and factors influencing such action. In the event that certain kinds of intestinal bacteria may destroy ascorbic acid while others do not, it would be advisable from the standpoint of proper human nutrition to attempt to control these offending organisms in the intestinal tract. Such an alteration of intestinal flora possibly might be brought about by diet. However, this investigation in itself is concerned with an *in vitro* study of certain kinds of intestinal bacteria and their action on ascorbic acid under different conditions.

[1] Contribution no. 316 of the Massachusetts Agricultural Experiment Station, Amherst, Mass.

[2] Based on portions of a thesis presented by William B. Esselen, Jr., April, 1938, to the Faculty of the Graduate School of the Massachusetts State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

REVIEW OF LITERATURE

The literature contains very little information pertaining to bacteria and ascorbic acid. Einhauser (1937) found that patients with achlorhydria were not easily saturated with ascorbic acid administered by mouth, and he attributed this effect to the destructive action of an acid condition and of bacteria in the stomach and upper part of the intestine. This work corroborates that of Stepp and of Marin mentioned above. Gagyi (1936), and Gagyi and Ujsaghy (1936), found that certain bacteria, particularly the more virulent pathogens, possess a capacity for destroying ascorbic acid, and that the inability of these organisms to grow in a 0.2 per cent solution of this substance and their ability to destroy it run parallel. Hou (1936) reported that the destruction of vitamin C experimentally in distilled water or in tap water was caused partly by microorganisms.

The incorporation of ascorbic acid in culture media for the cultivation of anaerobes has been investigated to a limited extent. Ehrismann (1935) found that ascorbic acid in a dilution of 1:1000 favored the growth of obligate anaerobes even under aerobic conditions. Kligler and Guggenheim (1938) found that vitamin C used in culture media for *Clostridium welchii* reduced the oxidation-reduction potential of the medium sufficiently so that this organism would grow even in the presence of air or of oxygen. They also observed that the loss of ascorbic acid was less in a growing culture of *C. welchii* than in sterile control media.

Kligler, Leibowitz, and Berman (1937), in a study of the effect of ascorbic acid on toxin production by *Corynebacterium diphtheriae* in culture media, found that ascorbic acid was rapidly oxidized in culture media at a temperature of 30° to 37°C., but that it remained unoxidized longer in the presence of bacterial growth. They suggested that ascorbic acid may destroy by an oxidation-reduction reaction the toxin produced by this organism, and that the mechanism is similar to that of the toxin-destroying action of aldehydes in general.

Tkachenko (1936) appears to be the only one who has reported

studies on the ability of bacteria to reduce dehydroascorbic acid to ascorbic acid. He found that this conversion took place in cultures of *Lactobacillus bulgaricus*, *L. acidophilus*, and *L. leichmanni*.

Some work has been done relative to the effect of microbial growth on the vitamin C content of foods. Lepkovsky, Hart, Hastings, and Frazier (1925) found that *Streptococcus lactis* had no effect on the vitamin C content of orange and tomato juices. Bifano and Servazzi (1935) infected lemons with *Penicillium digitatum* and found that their vitamin C content was the same as that of non-infected lemons.

Esselen (1938) found that a number of different intestinal bacteria exerted a "protective action" on the oxidation of ascorbic acid. He suggested that this "protective action" might be correlated with bacterial growth activities. Kendall and Chinn (1938) reported an investigation on the decomposition of ascorbic acid by bacteria. As a result of their work it would appear that specific strains of bacteria, rather than bacterial "species" are able to ferment ascorbic acid. They also reported that certain bacteria growing in an ascorbic acid medium exhibit a "protective action," that is, actually retard the oxidation of ascorbic acid. They found, too, that glucose exerts a definite sparing action upon the fermentation of ascorbic acid by bacteria.

Thus, a review of the literature indicates that bacteria may be classified into three groups as regards the stability of ascorbic acid in their presence, namely: (1) those organisms which destroy ascorbic acid; (2) those which protect ascorbic acid from oxidation; and (3) those which have no influence on this substance.

EXPERIMENTAL WORK

Methods

Throughout this investigation cultural methods described below were employed in the study of the action of bacteria on ascorbic acid. The media used were physiological saline (0.85 per cent sodium chloride), and nutrient broth of the following composition:

"Bacto" beef extract	3.0 grams
"Bacto" peptone	5.0 grams
Carbohydrate (when used)	10.0 grams
Distilled water	1000.0 ml.
Reaction	pH 6.3 to 6.5

This medium was prepared and distributed in 50 ml. test tubes containing 30 ml. of medium, or in 250 ml. Erlenmeyer flasks containing 100 ml. of the medium, and autoclaved for 20 minutes at 15 pounds pressure. One-milliliter portions of a 24-hour nutrient broth culture of the organism studied were used for inoculation. An incubation temperature of 37°C. was used at all times. Pure crystalline ascorbic acid was employed, and the desired amount added to the medium in aqueous solution.

The ascorbic acid content of the medium was determined at the beginning and end of each experiment by a modification of the 2,6-dichlorophenolindophenol dye method of Tillmans as described by Bessey and King (1933). The actual method employed throughout this study consisted in placing 20.0 ml. of the culture mixture into a 50 ml. flask and adding 5.0 ml. of glacial acetic acid (sufficient to lower the pH value of the titration mixture below 2.0). The acidified solution was titrated immediately with a standardized solution of the 2,6-dichlorophenolindophenol dye. The titration was completed within two minutes in each case. Determinations were made in duplicate or in triplicate, and good checks were obtained. For convenience, all data are reported in terms of milligrams of ascorbic acid per 20.0 ml. of medium, and as the per cent of loss of ascorbic acid during the incubation period. In studying the effect of bacteria on ascorbic acid different incubation periods were used, and the ascorbic acid content of the mixture was determined at the beginning and at the end of each incubation period. Thus, it was possible to calculate the per cent loss of ascorbic acid as influenced by the various factors studied. Throughout most of this work much smaller concentrations of ascorbic acid have been used than were used by previous workers, because, under actual conditions existing in the intestinal tract, ascorbic acid is present in only relatively small amounts.

From a preliminary study it was found that ascorbic acid is

rapidly oxidized in uninoculated culture media. Consequently, it was decided to use a five-hour incubation period in studying the influence of bacteria on ascorbic acid because, on longer incubation, the ascorbic acid itself would be practically all destroyed without bacterial influence and it would be difficult to interpret the results as to the influence that bacteria might have on this oxidation. It was also found that, throughout a range of pH values from 3.85 to 9.40, the degree of acidity had no significant influence on the rate of oxidation of ascorbic acid under the conditions of the experiment. Thus, it would seem that the change in the reaction of the medium due to bacterial action would not be a factor in influencing the oxidation of ascorbic acid because this substance is rapidly oxidized throughout the normal pH range of bacterial growth.

The oxidation of ascorbic acid in nutrient and in carbohydrate broth inoculated with various intestinal bacteria

As a preliminary step in studying the destruction of ascorbic acid as influenced by bacterial growth, eleven strains of *Escherichia coli*, and one strain each of *Aerobacter aerogenes*, *Salmonella enteritidis*, *Salmonella pullorum*, *Salmonella aertrycke*, *Eberthella typhosa*, *Bacillus subtilis*, and *Proteus vulgaris* were studied with reference to their influence on ascorbic acid in nutrient broth, carbohydrate broth, and physiological saline by the method described above. The carbohydrates employed were glucose, galactose, lactose, maltose, sucrose, dextrin, and starch. The pH value of each culture was determined at the end of the incubation period by means of a Beckman pH meter. The data obtained are presented in table 1.

Ascorbic acid determinations on control cultures of the organisms with no ascorbic acid present gave no indication that ascorbic acid or ascorbic acid-like substances were synthesized by the bacteria. In no instance did the bacteria cause any greater destruction of ascorbic acid than that which occurred in the uninoculated control medium. Most of the organisms, particularly the *Aerobacter* and *Escherichia* strains, exerted a pronounced inhibitory action on the oxidation of ascorbic acid.

TABLE 1

Final pH of the medium and loss of ascorbic acid by oxidation in inoculated nutrient broth, carbohydrate broth, and physiological saline

Incubation five hours at 37°C.

ORGANISM	NUTRIENT BROTH		GLUCOSE BROTH		GALACTOSE BROTH		LACTOSE BROTH		MALTOSE BROTH		SUCROSE BROTH		DEXTRIN BROTH		STARCH BROTH		PHYSIOLOGICAL SALINE, PER CENT LOSS
	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	
Control	6.3	85	5.8	80	5.8	78	4.7	89	5.9	88	5.8	86	5.8	84	6.3	88	47
A. aerogenes (A101)	5.9	51	4.6	37	4.9	65	5.1	71	5.4	67	4.7	71	5.4	54	6.3	69	29
E. coli (Crooks)	5.7	56	4.4	28	5.1	28	5.3	51	5.4	40	5.6	66	5.3	35	6.3	72	31
E. coli (A1)	5.3	64	4.7	28	5.3	31	5.4	65	5.8	54	5.8	66	5.6	40	6.1	72	31
E. coli (A2)	5.7	68	4.7	30	5.2	42	5.8	72	5.8	71	5.8	74	5.7	42	6.3	73	35
E. coli (A3)	5.6	75	4.6	32	5.3	47	5.4	63	5.7	65	5.8	74	5.6	54	6.3	81	25
E. coli (A4)	5.7	67	4.6	18	5.3	42	5.3	71	5.7	52	5.8	74	5.6	48	6.1	71	33
E. coli (A11)	5.5	63	4.6	26	5.2	37	5.5	61	5.8	49	5.8	64	5.7	38	6.3	64	29
E. coli (A12)	5.7	67	4.7	26	5.2	38	5.4	61	5.8	61	5.8	63	5.7	37	6.0	73	27
E. coli (A20)	5.8	49	4.6	31	5.3	34	5.4	60	5.8	56	5.8	61	5.8	37	6.4	63	11
E. coli (A21)	5.5	62	4.8	31	5.6	33	5.4	70	5.8	58	5.8	61	5.8	29	6.2	64	27
E. coli (A25)	5.7	63	4.8	33	5.4	41	5.5	67	5.7	73	5.1	64	5.6	40	6.2	71	
E. coli (A30)	5.3	69	4.7	49	5.2	22	5.5	74	5.7	69	5.0	68	5.5	45	6.0	75	
S. pullorum	5.2	82	5.9	80	6.0	75	6.2	87	6.2	79	6.0	80	6.0	80	6.4	79	
S. aertrycke	5.7	57	4.8	58	5.4	34	6.2	88	6.0	65	6.0	66	5.9	42	6.3	56	
S. enteritidis	5.8	70	4.8	50	5.3	37	6.1	88	5.9	54	6.0	66	5.8	36	6.1	63	47
E. typhosa	6.0	78	5.0	50	5.4	66	6.1	83	5.8	79	5.5	71	5.8	62	6.3	80	38
P. vulgaris	5.9	65	5.8	65	6.0	66	6.2	67	6.2	71	6.1	80	5.9	47	6.3	77	
B. subtilis	5.8	80	6.0	83	6.0	73	6.1	70	6.2	85	6.1	78	6.1	81	6.2	81	42

The cultures of those organisms which grew most rapidly exerted the strongest inhibitory action on the oxidation of ascorbic acid. The presence of a readily fermentable carbohydrate in the medium still further enhanced the ability of the microörganisms to inhibit the oxidation of ascorbic acid. In physiological saline the organisms appeared to exert a slight inhibitory action on the oxidation of ascorbic acid in some cases, but, as with the nutrient broth, in no instance did they cause any destruction of this substance. It is also evident from table 1 that the degree of acidity produced by the bacteria under observation is not a factor in the ability of the bacteria to inhibit the oxidation of ascorbic acid. There appears to be no correlation between the acidity produced by an organism and its ability to inhibit the oxidation of ascorbic acid.

Influence of relative number of bacteria and their stage of growth on the oxidation of ascorbic acid

A test was conducted to determine the influence of the size of inoculum on the loss of ascorbic acid by *Escherichia coli*, *Aerobacter aerogenes*, and *Proteus vulgaris*. From table 2 it may be seen that, in general, as the size of the inoculum was increased the oxidation of ascorbic acid was decreased. These results indicated that the inhibitory effect of bacteria on the oxidation of ascorbic acid increased with the increase in the number of bacteria present. In the event that such is the case, older but still active cultures of bacteria which would contain large numbers of organisms should inhibit the oxidation of ascorbic acid to a greater extent than the relatively young cultures so far studied.

A series of experiments were carried out to test this hypothesis. Ascorbic acid was added to 5, 17, 24, 48, and 72-hour nutrient broth and glucose broth cultures of the organisms previously used. After five hours the loss of ascorbic acid was determined. The data so obtained are presented in table 3. The ability of all of the strains studied to inhibit the oxidation of ascorbic acid increased with the age of the cultures up to 24 hours, but beyond this time the cultures of the organisms appeared slowly to lose this property. The cultures exhibited their most marked inhib-

itory action towards ascorbic acid oxidation when they were in the stages of growth in which the maximum number of viable organisms was present in the medium (Jordan and Falk (1928)). Organisms in the glucose broth developed the power to inhibit the oxidation of ascorbic acid sooner than did duplicate cultures in nutrient broth.

Apparently, then, the actively growing bacteria bring about some change in the media which tends to inhibit the oxidation of

TABLE 2

Influence of the quantity of inoculum on the loss of ascorbic acid in nutrient broth incubated at 37°C. for five hours

ORGANISM	QUANTITY OF INOCULUM	ASCORBIC ACID PER 20 ML. NUTRIENT BROTH	LOSS IN FIVE HOURS
	ml.	*mgm.*	*per cent*
Control (start)	None	0.65	
Control (5 hours)	None	0.07	89
E. coli (Crooks)	0.1	0.07	89
	1.0	0.20	69
	5.0	0.45	30
	10.0	0.35	46
A. aerogenes	0.1	0.10	85
	1.0	0.15	77
	5.0	0.47	28
	10.0	0.60	8
P. vulgaris	0.1	0.07	89
	1.0	0.08	88
	5.0	0.08	88
	10.0	0.08	88

ascorbic acid. Several possibilities present themselves in explanation of what happens during bacterial growth which might inhibit the oxidation of ascorbic acid, namely: (1) the formation of un-ionized copper complexes with protein decomposition products, by which the catalytic power of small amounts of copper naturally present (Dunham (1938)) might be destroyed (Barron, Barron, and Klemperer (1936)); (Ettisch, Sachsse, and Beck (1931)); (2) a change in the oxygen tension of the medium and its saturation with other gases given off as a result of bacterial

TABLE 3

Influence of the age of culture on the rate of oxidation of ascorbic acid in nutrient broth

ORGANISM	AGE OF CULTURE*	PER CENT LOSS OF ASCORBIC ACID IN 5 HOURS AT 37°C.†	
		Nutrient broth	Glucose broth
	hours	*per cent*	*per cent*
A. aerogenes (A101)	0	56	36
	5		8
	17	8	3
	24	0	0
	48	24	
	72	17	
E. coli (Crooks)	0	37	19
	5	36	
	17	19	7
	24	0	0
	48	15	
	72	22	
E. coli (A2)	0	63	11
	5	48	
	17	17	6
	24	0	0
	48	17	
	72	20	
E. coli (A25)	0	67	37
	5	45	
	17	37	3
	24	0	14
	48	22	
	72	31	
P. vulgaris	0	85	80
	5	49	17
	17	11	3
	24	0	0
	48	11	
	72	26	
B. subtilis	0	90	88
	5	47	33
	17	20	0
	24	32	0
	48	23	
	72	69	

* Age of culture when ascorbic acid was added.

† Media contained 0.87 mgm. of ascorbic acid at start of incubation periods.

growth activity; and (3) the production of bacterial metabolic products which might prevent the ascorbic acid from being oxidized.

The influence of the concentration of ascorbic acid on its rate of oxidation and on the growth of bacteria

Several series of flasks each containing 100 ml. of glucose broth were prepared so as to contain ascorbic acid in concentrations approximating 1.0, 0.1, 0.01, and 0.001 per cent respectively. Inoculums of three strains of *Escherichia coli* and one strain each of *Aerobacter aerogenes*, *Proteus vulgaris*, and *Bacillus subtilis* were added separately to the different series of flasks for 18 hours. After incubation, plate counts were made from each flask in order to determine the influence of the concentration of ascorbic acid on the growth of the bacteria. Ascorbic acid determinations were also made so that the per cent of loss of this substance as influenced by its concentration and the presence of bacterial growth in the medium could be calculated. These data are summarized in table 4. From this information it is quite evident that bacterial growth is inhibited as the concentration of ascorbic acid in the medium is increased above certain concentrations, depending upon the kind of bacteria employed. However, with the slower growing organisms such as *Proteus vulgaris*[3] and *Bacillus subtilis*, concentrations of ascorbic acid up to 0.01 per cent and 0.1 per cent respectively appeared to stimulate growth.

At the beginning of these experiments the influence of the concentration of ascorbic acid on the pH values of the media was measured and was found to be as follows:

Glucose broth without ascorbic acid	pH 6.31
Glucose broth plus 0.001 per cent ascorbic acid	pH 6.31
Glucose broth plus 0.01 per cent ascorbic acid	pH 6.08
Glucose broth plus 0.1 per cent ascorbic acid	pH 4.60
Glucose broth plus 1.0 per cent ascorbic acid	pH 3.65

[3] While *Proteus vulgaris* is generally considered to be a rapidly growing organism, the strains employed by the author grew slowly in comparison with *Escherichia coli*.

TABLE 4

The influence of concentration of ascorbic acid upon its rate of oxidation and upon bacterial growth in glucose broth at 37°C.

ORGANISM	ASCORBIC ACID PER 20 ML. MEDIA	LOSS OF ASCORBIC ACID IN 18 HOURS	NUMBER OF ORGANISMS PER ONE ML. OF MEDIA AFTER 18 HOURS
	mgm.	*per cent*	
Control (start)	181.93		
	18.13		
	1.75		
	0.11		
Control (18 hours)	169.93	7	
	7.93	56	
	0.08	95	
	0.01	00	
A. aerogenes (A101)	181.90	0	10,500
	15.93	12	100,000,000
	1.37	22	100,000,000
	0.11	0	130,000,000
	None		130,000,000
E. coli (Crooks)	139.93	23	13,500
	17.73	2	23,400,000
	1.61	8	80,000,000
	0.09	18	170,000,000
	None		250,000,000
E. coli (A2)	181.00	0	30,000
	15.33	14	14,400,000
	0.93	47	200,000,000
	0.05	55	132,000,000
	None		250,000,000
E. coli (A25)	179.93	1	20,000
	16.73	8	12,000,000
	0.79	55	50,000,000
	0.03	73	86,000,000
	None		100,000,000
P. vulgaris	141.93	22	20,000
	13.13	28	7,800,000
	0.54	69	70,000,000
	0.02	82	128,000,000
	None		20,000,000
B. subtilis	169.93	7	1,600
	18.10	0	4,200,000
	0.27	85	43,000,000
	0.01	99	150,000,000
	None		800,000

The above information would suggest that the inhibition of bacterial growth observed as a result of increasing the concentration of ascorbic acid may be due to the increased hydrogen ion concentration of the media produced by the ascorbic acid.

The production of carbon dioxide by bacteria as a factor influencing the stability of ascorbic acid

In the event that the observed inhibitory action of bacteria on the oxidation of ascorbic acid as reported above might have been due to gases produced by the organisms, the influence of carbon dioxide and of hydrogen on ascorbic acid oxidation was studied. Eighteen different bacterial strains were inoculated into flasks of nutrient broth to which ascorbic acid was added according to the technique previously employed. The flasks were incubated in anaerobe jars for five hours at 37°C. in atmospheres of carbon dioxide and of hydrogen respectively. The results are presented in table 5. In comparing these results with those in table 1 it may be seen that, in general, the oxidation of ascorbic acid in an atmosphere of hydrogen is similar to that which takes place in nutrient broth under aerobic conditions. The carbon dioxide exerted an inhibiting effect on the oxidation of ascorbic acid to a degree parallel to that exerted by the bacteria when they were cultured in nutrient broth containing a readily fermentable carbohydrate. Thus, it would seem that the protective action of the bacteria on ascorbic acid may be due, at least in part, to the carbon dioxide which they produce. This possibility was investigated by measuring the quantities of total dissolved gas and of dissolved carbon dioxide which were present in the cultures as a result of bacterial metabolism. The manometric method of Van Slyke and Neill, as described by Hawk and Bergeim (1931) was employed for the measurement. The quantities of total gas and of carbon dioxide produced by the bacteria in the cultures were compared with the degree of oxidation of ascorbic acid in the medium as influenced by the bacteria present.

The results are given in table 6, from which it may be seen that the organisms which significantly inhibited the oxidation of

ascorbic acid also produced considerable carbon dioxide and total gas in comparison with those which did not retard the oxidation of ascorbic acid. Although these data show a correlation between carbon dioxide production by bacteria and their ability to inhibit

TABLE 5

Influence of atmospheres of carbon dioxide and hydrogen on the oxidation of ascorbic acid in nutrient broth inoculated with different intestinal bacteria in five hours at 37°C.

ORGANISM	IN ATMOSPHERE OF HYDROGEN		IN ATMOSPHERE OF CARBON DIOXIDE	
	Ascorbic acid per 20 ml. broth	Loss	Ascorbic acid per 20 ml. broth	Loss
	mgm.	*per cent*	*mgm.*	*per cent*
Control (start)	0.74		0.72	
Control (5 hours)	0.13	82	0.40	44
A. aerogenes (A101)	0.24	68	0.47	35
E. coli (Crooks)	0.32	57	0.42	42
E. coli (A1)	0.43	42	0.52	28
E. coli (A2)	0.30	59	0.53	26
E. coli (A3)	0.34	54	0.55	24
E. coli (A4)	0.32	57	0.60	17
E. coli (A11)	0.36	51	0.50	30
E. coli (A12)	0.31	58	0.55	24
E. coli (A20)	0.33	55	0.50	30
E. coli (A21)	0.35	53	0.50	30
E. coli (A25)	0.39	47		
E. coli (A30)	0.41	45		
S. pullorum	0.18	76		
S. aertrycke	0.33	55	0.40	44
S. enteritidis	0.32	57	0.60	17
E. typhosa	0.29	61		
P. vulgaris	0.19	74	0.50	30
B. subtilis	0.16	78	0.40	44

the oxidation of ascorbic acid, the evidence does not prove that the carbon dioxide is the only inhibiting factor involved.

In order to determine the rôle, if any, of carbon dioxide production by bacteria in the prevention of the oxidation of ascorbic

TABLE 6

The total amounts of dissolved gas and of carbon dioxide produced by bacteria in glucose broth in five hours at 37°C. and the oxidation of ascorbic acid under the same conditions

ORGANISM	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS	TOTAL VOL. OF GAS AS CC. PER 100 ML. OF BROTH	VOL. OF CARBON DIOXIDE PER 100 ML. OF BROTH
	mgm.	*per cent*		
Control (start)	0.75			
Control (5 hours)	0.10	86	2.1	0.0
A. aerogenes (A101)	0.46	39	12.8	4.7
E. coli (Crooks)	0.52	30	6.2	1.9
E. coli (A1)	0.55	26	8.8	3.4
E. coli (A2)	0.39	48	7.7	2.2
E. coli (A3)	0.45	37	9.0	1.9
E. coli (A4)	0.41	43	8.0	1.9
E. coli (A11)	0.47	35	9.6	2.3
E. coli (A12)	0.45	37	9.3	3.2
E. coli (A20)	0.49	29	8.8	2.1
E. coli (A21)	0.47	35	9.2	2.8
E. coli (A25)	0.43	42	8.8	2.3
E. coli (A30)	0.47	35	8.3	2.1
S. pullorum	0.12	84	2.5	0.0
S. aertrycke	0.37	50	8.2	1.8
S. enteritidis	0.40	46	9.3	1.9
E. typhosa	0.20	73	3.2	0.0
P. vulgaris	0.19	75	2.4	0.0
B. subtilis	0.11	85	2.0	0.0

TABLE 7

The influence of dissolved carbon dioxide on the rate of oxidation of ascorbic acid in uninoculated glucose broth in five hours at 37°C.

GROUP	DISSOLVED CARBON DIOXIDE PER 100 ML. BROTH	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS
	cc.	*mgm.*	*per cent*
Control (start)	0.0	0.77	
Control (5 hours)	0.0	0.08	90
Series A, plus CO_2	2.0	0.19	75
Series B, plus CO_2	4.6	0.30	61
Series C, plus CO_2	13.4	0.30	61

acid, an experiment similar to the last one was conducted, only in this case three series of an uninoculated medium containing

graduated amounts of dissolved carbon dioxide were used. Ascorbic acid was added to each flask and carbon dioxide dissolved in the medium by placing the flasks in anaerobe jars and adding carbon dioxide to the atmosphere of the jars. When the medium contained the desired amount of this gas (previously determined) the anaerobe jars containing the flasks of medium were incubated for five hours at 37°C. At the end of this period the ascorbic acid and carbon dioxide contents of each series were determined. The data presented in table 7 show that the presence of carbon dioxide in glucose broth did exert a small inhibitory effect on the rate of oxidation of ascorbic acid. However, this inhibitory effect was not as marked as that produced by bacteria which evolve carbon dioxide when grown in glucose broth. The uninoculated glucose broth used in the last experiment contained a greater concentration of dissolved carbon dioxide than was produced by bacteria under similar conditions. This fact indicated that carbon dioxide production by bacteria is not the only factor in the mechanism whereby they inhibit the oxidation of ascorbic acid.

Assuming that the carbon dioxide production by bacteria was one factor influencing their inhibitory action on the oxidation of ascorbic acid, another set of experiments was conducted in order to find out whether bacteria exert this effect by combinations of three factors, namely: (1) carbon dioxide production, (2) removal of oxygen from the medium, and (3) the formation of un-ionized copper complexes. In this experiment uninoculated glucose broth was used as the medium, and the flasks were incubated in anaerobe jars for five hours at 37°C. The influence of carbon dioxide was determined by adding the gas at 20 pounds pressure to the atmosphere in the anaerobe jars. The effect of the removal of oxygen from the medium by bacterial action was simulated by evacuating the anaerobe jars with a vacuum pump; and the formation of un-ionized copper complexes by the bacteria was duplicated by adding 0.03 gram of 8-hydroxyquinoline to each 100 ml. of medium. (It has been previously shown by Barron, Barron, and Klemperer (1936) that 8-hydroxyquinoline destroys

the catalytic action of small amounts of copper on the oxidation of ascorbic acid.) The results are presented in table 8.

Each of the above factors exerted a certain amount of inhibitory action on the oxidation of ascorbic acid, but when they were used in combinations of two together, or of all three together, their combined inhibitory effect was greater than the total inhibitory effect of these three factors, namely, carbon dioxide, vacuum, and 8-hydroxyquinoline, employed separately. In

TABLE 8

The inhibitory effect of carbon dioxide, vacuum, and 8-hydroxyquinoline on the oxidation of ascorbic acid in uninoculated glucose broth in 5 hours at 37°C.

EXPERIMENTAL GROUP	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS	INHIBITION OF ASCORBIC ACID OXIDATION*
	mgm.	*per cent*	*per cent*
Control (start)	0.79		
Control (5 hours)	0.10	87	
Control plus 8-hydroxyquinoline	0.12	85	2
Control plus vacuum	0.19	76	11
Control plus carbon dioxide	0.24	70	17
Control plus 8-hydroxyquinoline plus vacuum	0.32	60	27
Control plus 8-hydroxyquinoline plus carbon dioxide	0.47	40	47
Control plus vacuum plus carbon dioxide	0.47	40	47
Control plus vacuum plus carbon dioxide plus 8-hydroxyquinoline	0.69	13	74

* Note: The per cent inhibition of ascorbic acid oxidation refers to the difference between the per cent loss of ascorbic acid in the control medium and that in the control media plus the substance under consideration.

comparing these data with those previously obtained with bacteria it would seem that the ability of the various bacteria to inhibit the oxidation of ascorbic acid might be due to their ability to form un-ionized copper complexes, and to their production of carbon dioxide in, and their removal of oxygen from, the medium. It is not unreasonable to suspect that the intensity of the action of the above three mechanisms would vary with different species of bacteria and with their stage of growth. When studied separately the inhibitory action of carbon dioxide was greater

than that of either a vacuum or of 8-hydroxyquinoline. The 8-hydroxyquinoline was the least active in this respect, but when this substance was used in combination with carbon dioxide or with a vacuum the resultant inhibitory action was more pronounced.

If it is assumed that all bacteria in their growth in culture media are capable of forming un-ionized copper complexes, then bacterial species which produce carbon dioxide in their metabolic processes should exert a more marked inhibitory effect on the oxidation of ascorbic acid than those organisms which do not produce carbon dioxide and, also, the inhibitory effect of carbon dioxide-producing species should be greater than that of carbon dioxide alone when added to uninoculated culture media. Likewise, those species producing little or no carbon dioxide should exert a stronger inhibitory action when grown in an atmosphere of this gas. Also, species such as *Escherichia coli*, when they are grown in nutrient broth to which carbon dioxide is added, should exert an inhibitory effect similar to that which they exert when grown in glucose broth. The reduced oxygen tension of the medium in which the bacteria are grown, which was duplicated by the use of a vacuum, is another factor which should influence the ability of bacteria to inhibit the oxidation of ascorbic acid. However, this factor is likely to be variable due to the cultural conditions and the changing vigor of the bacterial growth.

If the data in tables 1, 5, 6, and 8 are compared it will be seen that the observations made in this investigation substantiate the above explanation of the mechanism by which bacteria inhibit the oxidation of ascorbic acid.

The influence of killed cultures of bacteria and of cell-free culture filtrates on the oxidation of ascorbic acid

It has been suggested that the inhibitory action of bacteria on the oxidation of ascorbic acid is due principally to the production of carbon dioxide and the reduced oxygen tension of the medium as a result of their metabolic activities. If such is the case it would seem that killed cultures of the bacteria, or the cell-free filtrates from such cultures, would not exhibit the inhibitory

action observed with actively growing cultures of bacteria. Three series of tests were conducted to investigate this hypothesis.

Twenty-four-hour cultures of *Escherichia coli* and of *Salmonella aertrycke* in glucose broth were filtered through Berkefeld filters. Ascorbic acid was added to these culture filtrates and they were incubated. At the end of five hours the per cent of loss of ascorbic acid was determined. Also, twenty-four-hour cultures of *Aerobacter aerogenes*, *Escherichia coli*, *Proteus vulgaris*, and *Bacillus subtilis* were killed by 15 pounds steam pressure for 15 minutes, and by the addition of 4.0 ml. of 20 per cent phenol per culture, respectively. Ascorbic acid was added to the killed cultures, and at the end of a five-hour incubation period at 37°C. the per cent of loss of ascorbic acid was determined. In all cases it was found that the absence of actively growing bacteria, whether they were removed from the medium by filtration or were killed, resulted in the loss of the ability of the cultures to inhibit the oxidation of ascorbic acid significantly. Thus, further evidence is provided to show that in order to prevent the oxidation of ascorbic acid in culture media actively growing bacteria must be present.

The ability of bacteria to reduce dehydroascorbic acid

The only report which has been found in the literature pertaining to the reduction of dehydroascorbic acid by bacteria is that of Tkachenko (1936) who reported that such a reduction did take place in cultures of several species of "Lactobacilli." Dehydroascorbic acid was prepared according to the method of Kohman and Sanborn (1937) by oxidizing ascorbic acid in aqueous solution with iodine. Eleven strains of *Escherichia coli*, and one strain each of *Aerobacter aerogenes*, *Salmonella pullorum*, *Salmonella aertrycke*, and *Salmonella enteritidis* were studied with reference to their ability to reduce dehydroascorbic acid. The acid was added to 24-hour cultures of these organisms in glucose broth. At the beginning of the experiment the ascorbic acid in the medium was all present in the dehydro-form, but at the end of five hours two of the strains of *Escherichia coli* had reduced approximately 90 per cent of the dehydroascorbic acid to its

equivalent of ascorbic acid, as may be seen in table 9. Of the organisms studied only these two strains of *Escherichia coli* were capable of bringing about this reduction. Repeated tests furnished definite proof that the two strains of *Escherichia coli* were unique in this respect among the strains employed.

TABLE 9

The reduction of dehydroascorbic acid to ascorbic acid by 24-hour glucose broth cultures of bacteria in five hours at 37°C.

ORGANISM	ASCORBIC ACID PER 20 ML. OF BROTH	REDUCTION OF DEHYDROASCORBIC ACID TO ASCORBIC ACID
	mgm.	*per cent*
Control (start)	0.62*	
Control (5 hours)	0.00	0
A. aerogenes (A101)	0.04	6
E. coli (Crooks)	0.54	87
E. coli (A1)	0.58	94
E. coli (A2)	0.01	2
E. coli (A3)	0.01	2
E. coli (A4)	0.01	2
E. coli (A11)	0.01	2
E. coli (A12)	0.01	2
E. coli (A20)	0.01	2
E. coli (A21)	0.01	2
E. coli (A25)	0.01	2
E. coli (A30)	0.01	2
S. pullorum	0.01	2
S. aertrycke	0.01	2
S. enteritidis	0.01	2

* Ascorbic acid equivalent of dehydroascorbic acid.

SUMMARY

1. It has been found that certain bacteria, particularly members of the coliform group, inhibited the oxidation of ascorbic acid in culture media. The stronger inhibitory action was observed with the more actively growing cultures in which the largest numbers of bacteria were present.

2. Ascorbic acid was rapidly oxidized in uninoculated nutrient broth at 37°C., and this oxidation was retarded by the presence of carbon dioxide, by 8-hydroxyquinoline, and in the absence of oxygen.

3. It is suggested that growing bacteria inhibit the oxidation of ascorbic acid by certain combinations of three factors, namely: (1) the formation of un-ionized copper complexes whereby the catalytic action of the copper is destroyed, (2) the production of carbon dioxide with the subsequent saturation of the medium with it, and (3) the lowering of the oxygen tension of the medium. The most effective inhibitory action was obtained by a combination of all three of these factors together.

4. Two strains only of *Escherichia coli* were able to reduce dehydroascorbic acid to ascorbic acid, while the other bacteria studied did not appear to possess this ability.

5. The inhibitory action of bacteria on the oxidation of ascorbic acid was not observed in relatively large concentrations of this substance because the acidity of the medium was lowered below the point where effective bacterial growth could take place.

6. Killed bacterial cultures, or cell-free filtrates from living bacterial cultures, did not possess the inhibitory action on the oxidation of ascorbic acid that was observed with actively growing bacterial cultures.

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ON THE RELATION BETWEEN ASSIMILATION AND RESPIRATION IN SUSPENSIONS AND IN CULTURES OF ESCHERICHIA COLI

C. E. CLIFTON AND W. A. LOGAN

Department of Bacteriology and Experimental Pathology, Stanford University, California

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Until recently, it has been generally assumed that the conversion under highly aerobic conditions of relatively simple, non-nitrogenous compounds by washed suspensions of bacteria is limited to an oxidation of the substrate to carbon dioxide and water. Barker (1936), Geisberger (1936) and Clifton (1937) have shown that the oxidation does not proceed to completion, but that instead, a portion of the substrate is apparently assimilated by the cells. For example, the oxidative assimilation of acetate and of butyrate by *Pseudomonas calco-acetica* may be represented as

$$2CH_3COOH + 3\ O_2 = (CH_2O) + 3\ CO_2 + 3H_2O, \qquad (1)$$

and

$$2C_4H_8O_2 + 7\ O_2 = 3\ (CH_2O) + 5\ CO_2 + 5\ H_2O \qquad (2)$$

These equations indicate that approximately three-fourths of the oxygen required for the complete combustion of these substrates is consumed, with the production of carbon dioxide, water and a substance, or substances, having the empirical composition of a carbohydrate.

The oxidative assimilation of butyrate is of particular interest since the respiratory quotient (R.Q.) during the stage of rapid oxidation is approximately 0.68 while the theoretical R.Q. for complete combustion is 0.8. The rate of oxygen consumption abruptly decreases to a level near that of the control by the

time that three-fourths of the oxygen required for complete combustion has been consumed and the R.Q. increases to 0.94, a value characteristic of the respiration of the control suspension. However, in the presence of suitable concentrations of sodium azide (NaN_3) or of 2:4 dinitrophenol (α DNP) the oxidation proceeds to completion at a quite constant rate and the R.Q. throughout is equal to the theoretical value, 0.8.

The oxidative assimilation of lactic acid in washed suspensions and in cultures of *Escherichia coli* has recently been described by Clifton and Logan (1938). The oxygen uptake in a M/15 phosphate buffer of pH 7.1, proceeds at a rapid and quite constant rate until approximately two-thirds of the oxygen required for complete combustion has been consumed. Then the rate of oxygen consumption abruptly decreases and approaches the level of the substrate-free control, a behavior first noted by Cook and Stephenson (1928). These results, together with an observed R.Q. of 1.0, suggest that the oxidation of lactate is an oxidative assimilation process which may be represented as

$$CH_3CHOHCOOH + 2\,O_2 = (CH_2O) + 2CO_2 + 2\,H_2O \qquad (3)$$

In a synthetic medium containing lactate as the only source of carbon the rate of oxygen consumption also breaks abruptly when approximately two-thirds of the theoretical amount for complete combustion has been consumed, thus suggesting that equation 3 also represents the primary phase of assimilation in cultures of *E. coli*. Furthermore, in buffered suspensions of *E. coli* the oxidation of lactate proceeds to completion in the presence of critical concentrations of NaN_3 or of α DNP. The addition of these agents to the culture medium proves to be inhibitory to growth.

The studies to be reported here deal primarily with the oxidative assimilation of acetate, pyruvate, glycerol, fumarate, succinate and glucose by washed suspensions of *E. coli* and of certain of these substrates in cultures of the same organism.

EXPERIMENTAL

The strain of *Escherichia coli* (K-12) employed in these studies was grown on nutrient agar at 30°C.; after 20 hours the growth

was washed off with distilled water and centrifuged. The organisms were washed twice by centrifugation and finally suspended in distilled water. This stock suspension showed no marked decrease in metabolic activity on standing in the refrigerator for several days. It was diluted as needed with phosphate buffer to a final concentration of M/15 and a pH of 7.1 unless otherwise stated. Stock solutions of the various substrates were carefully prepared, neutralized to pH 7.1 if necessary and sterilized by filtration through Jena sintered glass filters ("G 5 on 3").

A synthetic medium (Clifton, Cahen and Morrow, 1936) containing ammonium chloride as the source of nitrogen and one organic compound as the source of carbon was employed in the studies on the relation between respiration and assimilation in actively proliferating cultures of *E. coli*. In all studies reported the synthetic medium was rather heavily inoculated with a 20-hour peptone culture. Control tests indicated that the inoculum did not contain an appreciable quantity of oxidizable material. The rates and extent of oxygen consumption and of carbon dioxide production at 30°C. were determined by the usual Warburg technic, a total volume of 2.2 ml. of liquid being employed in flasks of 16–17 ml. capacity shaken at a rate of 100 cycles per minute. NaN_3 or α DNP was always added to the bacterial suspension before the flasks were placed in the manometers. The substrate was tipped in from a side-arm of the flask at zero time, 15–20 minutes after the flasks were placed in the water bath. No marked differences in the results were observed when both poison and substrate were added to the bacterial suspension at zero time, the above procedure being adopted primarily for convenience.

The oxidative assimilation of acetate

The oxidation of acetate by washed suspensions of *E. coli* proceeds over a pH range of 5.6 to 7.7 in a manner similar to that previously reported for *Pseudomonas calco-acetica* (Clifton, 1937) and for *E. coli* (Cook and Stephenson, 1928). The rate of oxygen consumption remains at a relatively constant level,

irrespective of the initial concentration of acetate, over the range studied, M/50–M/400, until approximately three-fourths of the oxygen required for complete combustion has been consumed. At this time the rate of oxygen consumption decreases abruptly and approaches a level near that of the control suspension to which no acetate had been added. The R.Q. in phosphate buffer was approximately 1.0. No correction was applied for the blank respiration since it appears to be negligible in the presence of a readily utilizable substrate. If a correction for the blank respiration were applied, the oxygen consumed during the oxidation of the acetate would amount to somewhat less than the reported value of three-fourths of that required for complete combustion.

Krebs (1937) has reported that the oxidation of acetate by *E. coli* in a saline-phosphate buffer of pH 6.5 goes to completion at a quite constant rate. Very little free carbon dioxide is formed in a saline-bicarbonate suspension in equilibrium with a 95 per cent oxygen-5 per cent carbon dioxide gas mixture during the initial stage of oxidation, while in the latter stages the ratio of carbon dioxide liberated to oxygen consumed is nearly 1.0. This observation suggested that the oxidation of acetate proceeds by stages. In the first stage only bicarbonate is set free, i.e., not more than one molecule of CO_2 is formed per molecule of acetate oxidized, while in the second stage carbon dioxide is set free.

We were unable to confirm these observations of Krebs, the oxidation of acetate by our strain of *E. coli* in Krebs saline-phosphate buffer mixtures of pH 6.5 to 7.5 going only about three-fourths to completion, even when the air in the Warburg flasks was replaced by oxygen. Also, no appreciable lag in carbon dioxide production was observed in the saline-bicarbonate buffer in equilibrium with a 95 per cent oxygen-5 per cent carbon dioxide gas mixture and the rate of carbon dioxide liberation was always approximately equal to the rate of bicarbonate formation in the studies on the kinetics of the oxidation of acetate.

Since the amount of acetate added to the bacterial suspension

at zero time was known and the amounts of oxygen consumed and of carbon dioxide produced were determined, it is possible to deduce the empirical composition of the other product, or products, just as the synthesis of a carbohydrate is deduced from the value of the photosynthetic quotient. Applying this mode of reasoning to the experimental data for the oxidation of acetate it is apparent that the balanced equation for this reaction may be represented as

$$2\ CH_3COOH + 3\ O_2 = (CH_2O) + 3\ CO_2 + 3\ H_2O \qquad (4)$$

That is, the acetic acid is oxidized to form a carbohydrate, carbon dioxide and water. It is true that no direct evidence is available that a carbohydrate is actually formed and stored by *E. coli* but Geisberger (1936) has demonstrated an increase in the volutin content of *Spirillum serpens* in similar experiments with calcium lactate as the substrate. Also the fact that the oxidation of lactate in either buffered suspensions or in actively proliferating cultures proceeds only two-thirds to completion lends indirect evidence for the hypothesis of oxidative assimilation by actively respiring but non-proliferating cells. Furthermore, possible explanations for the oxidation stopping short of completion have been shown to be untenable (Clifton, 1937). Only formate, of all the compounds studied, is oxidized completely to carbon dioxide and water by *E. coli*.

The oxidation of acetate in phosphate buffer or saline-bicarbonate proceeds to completion in the presence of M/400 NaN_3 or of M/2000 α DNP in accordance with the equation

$$CH_3COOH + 2\ O_2 = 2\ CO_2 + 2\ H_2O \qquad (5)$$

NaN_3 or α DNP in the concentrations employed had no appreciable effect on the rate of oxygen consumption by the control suspension. Also the rate of oxygen consumption in the presence of concentrations of NaN_3 or α DNP just sufficient to prevent assimilation is not markedly different from the rate observed in the absence of the poisons. Still higher concentrations of these agents do, however, inhibit respiration.

The oxidation of three-carbon compounds

The oxidation of propionic acid proceeds in a manner similar to that described above for acetate. However, propionate is not as readily oxidized as acetate and the oxidative assimilation of this compound was not studied in detail. The oxidation of the hydroxy-propionic acid (lactic) has been described (Clifton and Logan, 1938) and appears to be an oxidative assimilation proceeding according to equation 3, two-thirds of the amount of oxygen (4 atoms per mol of lactate) required for complete combustion being consumed. The oxidation of the keto-propionic acid (pyruvic) proceeds in a manner similar to that described for lactate, with the exception that the amount of oxygen consumed amounts to three-fifths of that required for complete combustion. The oxygen consumed during the oxidation of pyruvate corresponds to three atoms of oxygen per molecule, thus agreeing with the observation of Stephenson (1928) that lactate passes through the stage of pyruvate during aerobic oxidation by loss of two atoms of hydrogen.

Pyruvate is somewhat unsatisfactory for quantitative studies since it is unstable in solution. The following data are therefore presented as tentative only. The R.Q. in a limited number of tests was 1.6 or higher during the first stages of oxidation and decreased to 1.36 to 1.38 by the time the oxidation reached three-fifths of completion. These results suggest that the oxidative assimilation of pyruvate may be approximately represented as

$$2\ CH_3COCOOH + 3\ O_2 = 2\ (CH_2O) + 4\ CO_2 + 2\ H_2O \qquad (6)$$

The oxidation of pyruvate by *E. coli* approaches completion in the presence of M/200 NaN_3 or M/2000 α DNP, as illustrated by typical results presented in figure 1, *A*. The rate of oxygen consumption is inhibited about 50 per cent in the presence of M/2000 α DNP while no marked inhibition is observed in the presence of concentrations of α DNP less than M/4000. However, the extent to which the oxidation of pyruvate approaches completion decreases with decreasing concentration of α DNP being approximately 85, 75 and 65 per cent, respectively, in M/4000, M/8000 and M/16,000 solutions of α DNP.

The amounts of oxygen consumed during the growth of *E. coli*

in the synthetic medium containing pyruvate as the carbon and energy source were approximately equal to three-fifths of the amounts required for complete oxidation of pyruvate to carbon dioxide and water. Typical time-growth, time-oxygen consumption and time-carbon dioxide production relationships are presented in figure 1, *B*. Values for the oxygen consumption and carbon dioxide production per ml. per hour and per cell per

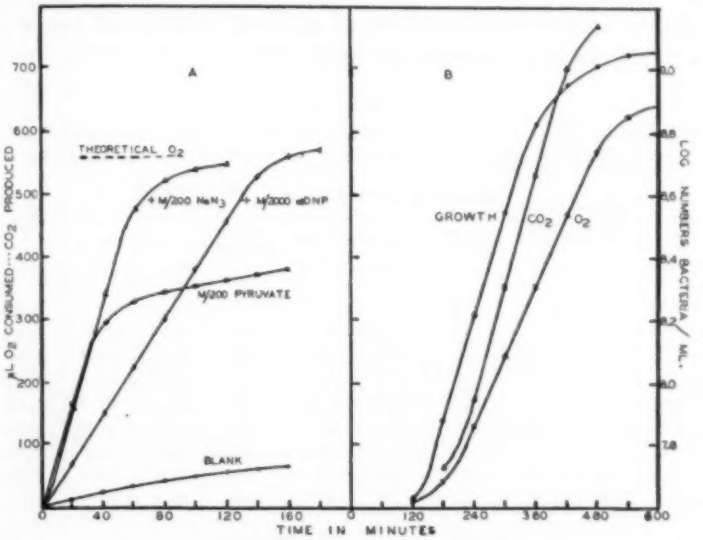

FIG. 1. THE OXIDATION OF PYRUVATE BY E. COLI

(*A*) the influence of NaN_3 and of αDNP on the oxidation of 2 ml. of M/200 pyruvate. (*B*) typical time-growth, time-oxygen consumption and time-carbon dioxide production relationships observed in 2 ml. of a synthetic medium, M/100 with respect to pyruvate.

hour (computed by Buchanan's (1930) formula), together with the observed R.Q.'s are presented in table 1.

The oxidation of glycerol by *E. coli* was studied in considerable detail, as this substrate is very stable under ordinary conditions and can readily be obtained in a pure state. Typical results on the rate and extent of oxidation of different concentrations of glycerol are presented in figure 2. In figure 2, *A*, the total oxygen consumption is plotted against time while in 2, *B*, the

oxygen consumed per ten-minute interval is plotted against the corresponding time. This latter method of plotting the results clearly shows the marked break in the rate of oxygen consumption which is observed by the time the oxidation reaches four-sevenths of completion as shown in figure 2, *A*. It can readily be seen in this latter figure that the break occurs around four-sevenths of completion irrespective of the initial concentration of glycerol over the range reported. The break is also observed in the same region in higher concentrations of glycerol, over a pH range of 5.6 to 7.7, or at a temperature of 37.5°C.

TABLE 1

Influence of the age of a culture of E. coli on the rate of oxygen consumption and of carbon dioxide production per ml. of a M/100 pyruvate-inorganic medium

TIME	NUMBER OF BACTERIA AT END OF TIME INTERVAL	MICROLITERS OF O_2 CONSUMED		MICROLITERS OF CO_2 PRODUCED		R.Q.
		per ml. per hour	per cell per hour	per ml. per hour	per cell per hour	
minutes						
0–120	4.2×10^7	5		7		1.40
120–180	7.6×10^7	18	31.3×10^{-8}	28	48.7×10^{-8}	1.55
180–240	19.5×10^7	40	31.5×10^{-8}	55	43.4×10^{-8}	1.38
240–300	36.8×10^7	58	21.1×10^{-8}	88	31.1×10^{-8}	1.52
300–360	66.8×10^7	59	11.7×10^{-8}	90	18.5×10^{-8}	1.52
360–420	91.2×10^7	56	7.1×10^{-8}	75	9.6×10^{-8}	1.34
420–480	105.0×10^7	50	5.3×10^{-8}	48	4.9×10^{-8}	0.96
480–540	113.0×10^7	31	2.7×10^{-8}	29	2.6×10^{-8}	0.93
540–600	116.0×10^7	8	0.7×10^{-8}	9	0.8×10^{-8}	1.12
Total		325		429		1.32

The theoretical R.Q. for the oxidation of glycerol is 0.86—while the R.Q. observed in phosphate buffer was in the neighborhood of 0.75 in the early stages of oxidation and approached a value of 0.80 by the time the oxidation approached four-sevenths of completion. A similar behavior was observed in bicarbonate buffer in equilibrium with a 5 per cent carbon dioxide-95 per cent oxygen gas mixture, the R.Q. up to the break being approximately 0.80. No change (outside of the experimental limits of error) in the concentration of bicarbonate was observed during the oxidation of glycerol, thus ruling out the possibility of carbon dioxide

being bound, or released due to the formation of an acidic by-product of oxidation.

The above results indicate that the oxidative assimilation of glycerol by *E. coli* may be approximately represented as

$$2\ C_3H_8O_3 + 4\ O_2 = 3\ (CH_2O) + 3\ CO_2 + 5\ H_2O \qquad (7)$$

This equation shows that four-seventhes of the amount of oxygen required for complete combustion is consumed with the pro-

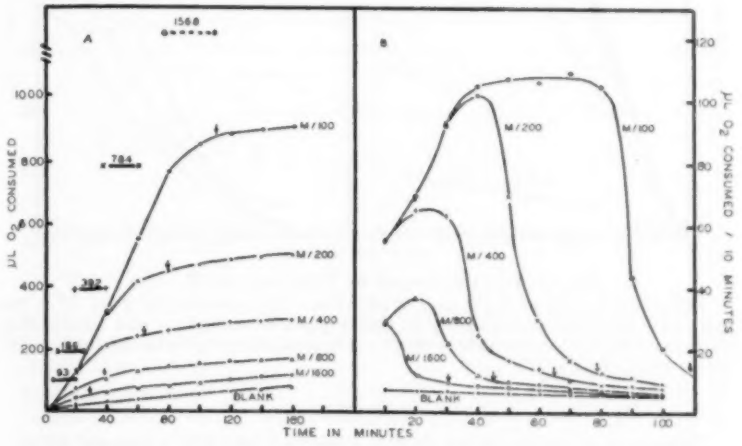

FIG. 2. THE OXIDATION OF 2 ML. OF DIFFERENT CONCENTRATIONS OF GLYCEROL BY E. COLI

Arrows indicate time at which glycerol is assumed to be decomposed, the total oxygen required for complete combustion being indicated by the heavy horizontal lines.

duction of 1.5 mols of a carbohydrate of empirical composition CH_2O per mol of glycerol utilized. The R.Q. of 0.75 postulated by this equation is in fair agreement with the observed R.Q.'s reported above.

The oxidation of glycerol approaches completion in the presence of M/200 NaN_3 or of M/2000 α DNP. Typical results are presented in figure 3, *A*. The observed R.Q.'s are in the neighborhood of the theoretical value, 0.86, and are recorded in table 2.

The amounts of oxygen consumed during the growth of *E. coli*

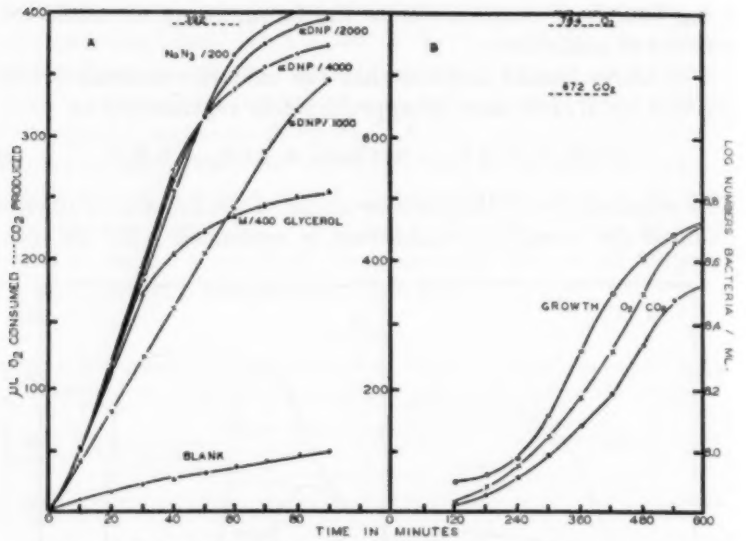

FIG. 3. THE OXIDATION OF GLYCEROL BY E. COLI

(*A*) the influence of NaN_3 and of αDNP on the oxidation of 2 ml. of M/200 glycerol. (*B*) typical time-growth, time-oxygen consumption and time-carbon dioxide production relationships observed in 2 ml. of a synthetic medium, M/200 with respect to glycerol.

TABLE 2

Oxygen consumption, carbon dioxide production and R.Q.'s observed during the oxidation of 2 ml. of M/200 glycerol by E. coli

	MINUTES	O_2	CO_2	R.Q.
Glycerol only	30	225	170	0.75
	60	381	295	0.77
	90	425	358	0.84
	120	463	370	0.80
M/200 NaN_3	20	175	136	0.78
	50	481	411	0.86
	70	617	518	0.83
	90	690	578	0.84
M/2000 αDNP	30	145	125	0.86
	60	337	274	0.81
	90	551	473	0.86
	120	676	589	0.87

in the synthetic medium containing glycerol as the carbon and energy source were approximately equal to four-sevenths of the amounts required for the complete oxidation of glycerol to carbon dioxide and water, thus again illustrating the similarity between assimilation in non-proliferating suspensions and in cultures of bacteria. Typical time-growth, time oxygen consumption and time-carbon dioxide production relationships observed in a M/200 glycerol medium are presented in figure 3, *B*, and in table 3.

TABLE 3

Influence of the age of a culture of E. coli on the rate of oxygen consumption and of carbon dioxide production in a M/200 glycerol-inorganic medium

TIME	NUMBER OF BACTERIA/ML. AT END OF TIME INTERVAL	MICROLITERS OF O_2 CONSUMED		MICROLITERS OF CO_2 PRODUCED		R.Q.
		per ml. per hour	per cell per hour	per ml. per hour	per cell per hour	
minutes						
0–120	7.9×10^7	5		4		0.80
120–180	8.3×10^7	15	14.7×10^{-8}	12	11.7×10^{-8}	0.80
180–240	8.9×10^7	17	21.5×10^{-8}	13	16.4×10^{-8}	0.76
240–300	13.0×10^7	24	22.2×10^{-8}	17	15.7×10^{-8}	0.71
300–360	20.5×10^7	28	16.7×10^{-8}	21	12.6×10^{-8}	0.75
360–420	30.2×10^7	36	14.5×10^{-8}	28	11.3×10^{-8}	0.75
420–480	40.3×10^7	49	13.9×10^{-8}	39	11.1×10^{-8}	0.79
480–540	48.8×10^7	44	9.9×10^{-8}	37	8.3×10^{-8}	0.84
540–600	52.5×10^7	14	2.8×10^{-8}	12	2.4×10^{-8}	0.86
Total		232		183		0.79

The oxidation of fumarate and succinate

The oxidation of fumarate by *E. coli* tends to go approximately two-thirds to completion before the rate of oxygen consumption falls to a level near that of the control without added substrate. Since the observed R.Q. was approximately 1.50 (theoretical 1.33) the oxidative assimilation of fumarate may be represented as

$$C_4H_4O_4 + 2\,O_2 = (CH_2O) + 3\,CO_2 + H_2O \qquad (8)$$

During the oxidation of succinate by *E. coli* one atom more of oxygen is consumed than during the oxidation of fumarate. This difference in oxygen consumption is in agreement with the

observations of Quastel and Wheatley (1931) that the normal course of biological oxidation of succinate proceeds through fumarate. The observed oxygen consumption of approximately five-sevenths of the theoretical and an R.Q. of 1.20 (theoretical 1.14) suggests that the oxidative assimilation of succinate may be represented as

$$C_4H_6O_2 + 2\ 1/2\ O_2 = (CH_2O) + 3\ CO_2 + H_2O \qquad (9)$$

The oxidation of fumarate, succinate and related compounds will be reported in detail at a later date. In a limited number of experiments the presence of NaN_3 or of α DNP appears to inhibit the oxidation of fumarate and succinate to a much greater extent than that of the other substrates reported in this paper. For example, the oxidation of succinate by *E. coli* was inhibited almost 50 per cent by M/16,000 α DNP, although the oxidation did tend to approach completion under these conditions, while the oxygen consumption in the presence of M/2000 α DNP was only approximately equal to that of the control suspension. This marked inhibitory action of NaN_3 or of α DNP on the oxidation of fumarate and succinate suggests that the fumarate-succinate system does not play an important rôle as a respiratory catalyst (hydrogen carrier) in the oxidation of acetate, propionate, lactate, pyruvate or glycerol by *E. coli* under aerobic conditions. However, the possibility remains that the fumarate-succinate system may be involved in the assimilatory process.

The oxidation of glucose

The oxidation of glucose by suspensions of *E. coli* goes approximately one-half to completion, in agreement with the observation of Cook and Stephenson (1928). Krebs (1937) has reported that the oxidation of glucose by *E. coli* goes 70 per cent to completion at 40°C. and a pH of 6.8. However, inspection of his data indicates that a marked break in oxygen consumption occurs in the neighborhood of 50 per cent of completion. Since the R.Q. observed during the oxidation of glucose is 1.0, the assimilatory process may be represented as

$$C_6H_{12}O_6 + 3\ O_2 = 3\ (CH_2O) + 3\ CO_2 + 3\ H_2O \qquad (10)$$

The oxidation of glucose tends to approach completion in the presence of M/400 NaN_3 or M/2000 α DNP. In a typical experiment 664 μl. of oxygen were consumed and 659 μl. of carbon dioxide were produced in 160 minutes in the presence of M/2000 α DNP while in the same time the gaseous exchange in the absence of this agent was only 439 μl. of oxygen and 429 μl. of carbon dioxide. The oxygen consumption and carbon dioxide production in the latter case had reached a level near that of the control when approximately 340 μl. of oxygen had been consumed.

TABLE 4

Influence of the age of a culture of E. coli on the rate of oxygen consumption in a M/200 glucose-inorganic medium

TIME	NUMBER OF BACTERIA/ML. AT END OF TIME INTERVAL	MICROLITERS OF O_2 CONSUMED	
		per ml. per hour	per cell per hour
minutes			
0–120	6.5×10^7	10	
120–180	13.6×10^7	21	21.7×10^{-8}
180–240	32.3×10^7	34	15.7×10^{-8}
240–300	63.1×10^7	53	11.4×10^{-8}
300–360	92.9×10^7	80	10.4×10^{-8}
360–420	106.7×10^7	41	4.1×10^{-8}
420–480	114.3×10^7	11	1.0×10^{-8}
480–540	117.5×10^7	4	0.3×10^{-8}
Total		254	

A marked break in the rate of oxygen consumption during the growth of *E. coli* in the synthetic medium plus glucose was noted by the time that the oxidation had proceeded to between thirty-five to forty per cent of completion. Typical results of oxygen consumption and of growth in the glucose medium are recorded in table 4, carbon dioxide values being omitted as they are identical with the values reported for oxygen within the limits of experimental error. Glucose could no longer be detected in the medium immediately after the marked break in oxygen consumption was observed.

Since the concentration of phosphates was higher in the sus-

pensions than in the cultures, it was deemed advisable to repeat the studies with glucose as the source of energy and of carbon for growth. Accordingly three parts of the inorganic medium were diluted with one part of M/7.5 phosphate buffer of pH 7.1. The rate of oxygen consumption in this phosphate rich medium was approximately equal to that observed in the normal medium, but the marked break in oxygen consumption was observed somewhat nearer 50 per cent of completion. This effect remains unexplained, but preliminary tests show that it is not due to a change in pH of the medium. No appreciable difference was observed in the extent of oxidation of pyruvate or of glycerol in the normal and in the phosphate-rich medium.

DISCUSSION

The data presented in this paper indicate that the oxidation of a number of organic compounds by suspensions of *E. coli* is not carried to completion, but that instead a portion of the compound is assimilated by the cells, probably in the form of a carbohydrate. Barker (1936), and Geisberger (1936), have reached similar conclusions in regard to the oxidation of a variety of compounds by the colorless alga, *Prototheca zopfii* and various species of the genus *Spirillum*, respectively, while Benoy and Elliott (1937) have reported the synthesis of carbohydrate from a number of organic compounds by rat tissues under conditions similar to those prevailing in manometric experiments. Brück (1933) has demonstrated that iodoacetate inhibits the formation of glycogen from glucose or ethyl alcohol by yeast. This poison has also been shown to inhibit oxidative assimilation by *Pseudomonas calco-acetica* and *Escherichia coli* (Clifton, 1937). A number of other reports in the literature also demonstrate that the oxidation of various substrates is not carried to completion by suspensions of bacteria or by tissue slices. Thus, it becomes apparent that the study of respiration by the manometric technic does not necessarily separate assimilatory and dissimilatory processes.

Winzler (1938) has reported that the heat production during the oxidation of glucose and of acetate by yeast amounts to only 26.65 and 58.7 per cent respectively, of the theoretical

amounts for complete combustion to carbon dioxide and water while the heat production during the fermentation of glucose is 70.5 per cent of the theoretical. Winzler also reached the conclusion that carbohydrate was assimilated during respiration and calculated that the free energy efficiency of this assimilatory process was only 2.9 and 12.2 per cent, respectively, during the oxidation of glucose and of acetate. He also demonstrated that the oxidation of acetate proceeds to completion in the presence of suitable concentrations of α dinitrophenol.

The experiments reported in this paper leave no doubt that it is possible to poison the cells selectively in such a way that synthetical processes are prevented while at the same time respiration proceeds with complete oxidation of the substrate to carbon dioxide and water. This has been proved by the fact that with suitable concentrations of sodium azide or of α dinitrophenol the oxygen consumption and carbon dioxide production approach the theoretical values for complete oxidation before a marked break in the rate of respiration is observed.

Regardless of the initial concentration or total quantity of substrate, the oxygen consumed before the marked break in rate of consumption is observed is always a constant proportion of the total amount required for the oxidation of a given substrate. Therefore, the ratio of synthesis to oxidation must be constant for a given system. This suggests that there is a close connection between dissimilation and assimilation, possibly of the nature of coupled reactions for, as pointed out by Borsook (1935) (see also Clark, 1938), in order for synthesis to occur, i.e., for work to be done by a spontaneous reaction, there must be a simultaneous reaction involving an increase in free energy, and these two processes must be coupled in such a manner that electrons of hydrogen freed in one reaction are transferred to the other. Borsook also pointed out that the reduction of pyruvate to lactate (synthesis of lactate) by *Escherichia coli* in the presence of formate can be blocked by the addition of toluol, but that the reaction can proceed provided an appropriate artificial "carrier" between the two enzyme systems is added.

The inefficient utilization of energy by bacteria and other tissues may be explained as due to the stoichiometric relationship between the free-energy-yielding and the free-energy-requiring coupled reactions, the efficiency of synthesis being dependent upon the relative values of the free energies of the two halves of the reaction. Since the free energies of the assimilatory processes considered are much smaller than the free energies of the oxidations to carbon dioxide and water—that is the substrates are nearer in chemical potential to the cell material than to their oxidation products—there is necessarily much loss of energy. This is demonstrated by the fact that the same amount of assimilation occurs during the oxidation of lactate and pyruvate, or of succinate and fumarate, although the free energy of lactate or of succinate is greater than that of pyruvate or fumarate, respectively. This suggests that assimilation may involve the intermediate products of oxidation rather than the initial substrate.

It is true that the manner of action of NaN_3 or of α DNP in blocking the assimilatory process is still obscure. Experiments with methylene blue as the hydrogen acceptor indicate that NaN_3 does not inhibit the dehydrogenase systems of *E. coli*. Green, Needham and Dewane (1937) are of the opinion that synthesis of carbohydrate from lactate in muscle takes place in the following manner: a trace of phosphoglycerate will react with lactate to produce triosephosphate and pyruvate; this reaction will proceed if the triosephosphate is continually removed by condensation to hexosediphosphate and then to glycogen and phosphate. Given an energy source, the pyruvate formed in the oxido-reduction reaction could be phosphorylated and its conversion into a further supply of phosphoglycerate to react with lactate would follow.

The principle of carbohydrate formation by *E. coli* could possibly be a dehydrogenation of a portion of the substrate with a transfer of this hydrogen by means of a carrier such as coenzyme to phosphoglycerate, the reduced form of the latter being removed by condensation and complex carbohydrate formation. The formation and phosphorylation of pyruvate or related compounds

during the oxidation of the substrate would serve as a continual source of phosphoglycerate to enable the reaction to continue, energy being supplied by coupled reactions during which a portion of the substrate is oxidized to carbon dioxide and water. It has been demonstrated that iodoacetate, an agent shown to block assimilation by *Pseudomonas calco-acetica* and *Escherichia coli*, will block the conversion of triosephosphate into phosphoglycerate. Possibly NaN_3 and α DNP may also exert an inhibitory action in the chain of reactions leading to stored carbohydrate, thus allowing the oxidation to proceed to completion. Studies are in progress to determine if assimilation, and the blocking of assimilation does proceed as postulated above.

It has been demonstrated that the oxidative assimilation of lactate, pyruvate, glycerol or of glucose as observed in buffered suspensions of *E. coli* appears to be the primary assimilatory processes in actively proliferating cultures of this organism. We may therefore postulate that the general assimilatory process may be represented as:

$$\text{Substrate} + O_2 \text{ yielding energy} = \text{assimilated material} + CO_2 + H_2O$$

much as

$$CO_2 + H_2O + \text{radiant energy} = (CH_2O) + O_2$$

represents the assimilatory process in the green plant.

SUMMARY

It has been shown that the oxidation of acetate, propionate, lactate, glycerol, fumarate, succinate and glucose by washed suspensions of *Escherichia coli* in phosphate or bicarbonate buffers is not carried to completion. The results suggest that a portion of the substrate is assimilated as carbohydrate by the cells, the amount assimilated being dependent on the nature of the substrate and independent of its concentration.

Indirect evidence of this assimilation is presented in studies on the growth of *Escherichia coli* in a synthetic medium, the same relation between the extent of oxidation and assimilation being observed in actively proliferating cultures as in washed suspensions of this organism.

The respiratory and assimilatory processes appear to be closely connected although it is possible to block the assimilatory process and bring about a complete oxidation of the substrate by adding suitable concentration of sodium azide or of α dinitrophenol to the bacterial suspension.

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THE EXTRACELLULAR PROTEOLYTIC SYSTEM OF CLOSTRIDIUM PARABOTULINUM

S. S. ELBERG AND K. F. MEYER

George Williams Hooper Foundation for Medical Research and Department of Bacteriology, University of California, Medical School, San Francisco, California

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The enzyme complements of the proteolytic anaerobic bacteria have been widely investigated, but strangely enough *Clostridium botulinum* has been neglected in this respect. In view of the importance of this organism both theoretically and practically, as a producer of a potent neurotoxin, we have attempted to determine the nature and properties of the various enzymes which catalyze proteolysis by this organism and the relation of these enzymes to toxin production.

Wagner, Meyer and Dozier (1925) stated that the gelatinase which was produced in cultures of *C. botulinum* was influenced by the carbohydrate concentration of the medium and they observed a more rapid disappearance of the gelatinase in the presence of carbohydrate than in its absence.

Snipe and Sommer (1928) in their work on purification of botulinus toxin found that a gelatinase, a peptidase, and a lipase were precipitated with the toxin by acidification of the supernatant liquid from cultures.

Maschmann (1927, 1938) has also made a study of the proteolytic enzymes of *C. botulinum*. He found that "there is a proteinase, an aminopolypeptidase and a dipeptidase secreted in the medium during growth." These enzymes were determined by the amino-group titration procedure. They were activated by M/250 cysteine, M/100 hydrogen cyanide and were unaffected by iodoacetic acid. Maschmann also found that cysteine was necessary before the proteinase would attack clupeine.

The objections, however, to using amino group or carboxyl group determinations for estimating proteinase activity have been clearly stated by Northrop (1932) and Anson (1932). "The splitting of peptide linkages which is what is measured by determination of the number of amino and carboxyl groups freed is due not only to proteinases but also to peptidases which cannot digest proteins. The estimation of the proteinases of anaerobes by Maschmann are thus estimations not of proteinase alone but of proteinases plus peptidases." (Anson, 1938).

EXPERIMENTAL

Northrop (1932) determined that the first effect of proteinases such as pepsin and trypsin on proteins (gelatin, casein and edestin) was to cause a rapid decrease in the viscosity of the protein. A measure of the viscosity changes during the first few minutes of contact between "enzyme solution" (culture supernatant) and protein will therefore give an accurate estimate of the true proteinase concentration.

The gelatin and casein solutions were prepared as recommended by Northrop (1932) for standard methods of peptic digestion. The actual viscosimetric determination of proteinase also followed Northrop's procedure (1932) with minor alterations.

The equation which was used to evaluate culture fluids for proteinase in this method was that suggested by Northrop (1932):

$$N = \frac{ts}{t\mathrm{H_2O}} - 1$$

where N = the specific viscosity of the protein solution, ts = time of outflow of protein solution, $t\mathrm{H_2O}$ = time of outflow of water from same viscosimeter.

The values of N were then plotted against the time elapsed after the enzymes and substrate solutions were mixed. The percentage change in viscosity for a given period was then interpolated from the curves. The zero point was determined by extrapolating on the curve obtained with a portion of enzyme solution previously boiled.

The per cent changes obtained by interpolation for a group of dilutions were averaged and the mean value determined for a digestion period of thirty minutes. It will be seen from the data below that the per cent change caused by different dilutions of culture fluid varied directly with the reciprocal of the dilution used. The error determined with a time period of thirty minutes was within 5 per cent. When the period was taken at twenty minutes the error was consistently larger and therefore thirty minutes was chosen as the uniform point of calculation. (See fig. 1.)

It is obvious from the data that the method allows definite appraisal of the proteinase content of a supernatant fluid.

TABLE 1

Per cent change caused in viscosity of 2.5 per cent gelatin solution, pH 7.0 by various amounts of proteinase of C. botulinum

Temperature of reaction 35.5° C.

DIGESTION TIME	PER CENT CHANGE IN VISCOSITY				VALUE OF K				MEAN VALUE OF K	DILUTION RECIPROCAL CALCULATED MEAN K PER CENT CHANGE			
	Dilution reciprocal												
	2	3	4	5	2	3	4	5		2	3	4	5
minutes													
20	55.3	37.5	25.8	21.4	110.6	112.5	113.2	107	110.8	2.00	2.95	4.29	5.17
30	67.6	44.6	33	26.7	235.2	133.8	132	133.5	133.6	1.97	2.99	4.04	5.00

However there are certain points limiting the interpretation of any viscosity change of a protein by such an "enzyme solution." The most important is that by this method we have probably measured only one type of proteinase, "gelatinase," and have not measured the total proteinase activity. Other proteinases such as that responsible for milk clotting should also be investigated, not only to determine their presence but also to check the validity of the viscosimetric method. The investigation of the milk-clotting power of culture fluids of *C. parabotulinum* on purified enzyme preparations will form the subject of a subsequent paper.

It was next imperative that certain properties of the proteinase

be defined in order to determine the set of conditions under which it could be routinely evaluated in the future and, if possible, its relation to other proteinases.

Experiment 1. Relation between proteinase activity and pH

Isoelectric gelatin was adjusted to the desired pH with HCl or NaOH. The supernatant fluids from forty-eight-hour-old cultures were collected and brought to the proper pH value

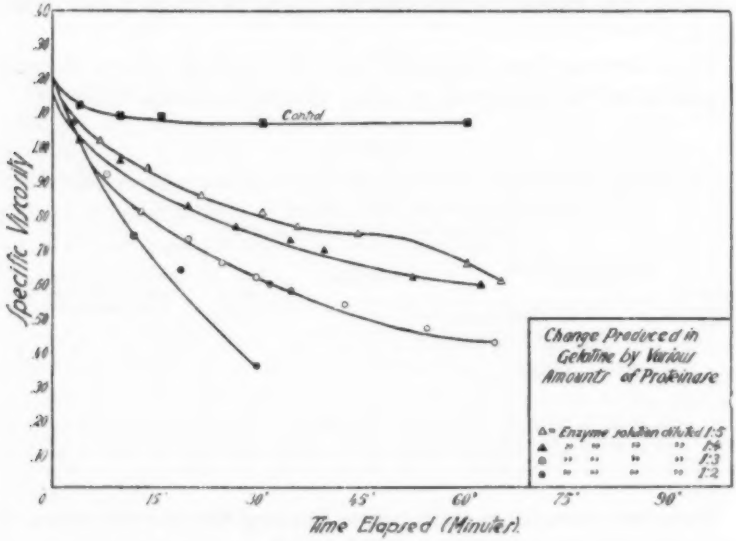

FIG. 1

before addition to the substrate. All pH values were controlled electrometrically.

The greatest change in the viscosity of a gelatin solution was obtained in thirty minutes at pH 7.0. In this respect the proteinase of *C. botulinum* differs from the animal proteinases, since the optimum pH for pepsin is about 2.5, for the cathepsins 3.7, for pancreatic proteinase 9, and for papain 7.4. On the other hand, the value obtained here agrees well with those for *Clostridium histolyticum*, *Clostridium sporogenes*, and *Clostridium*

welchii as reported by Weil and Kocholaty (1937), and other bacterial proteinases.

We did not at this time attempt to answer the question con-

TABLE 2

Summary of the pH-activity data

pH	PER CENT CHANGE IN 30 MINUTES
8.0	59
7.0	71.5
6.0	62.6
4.95	11.4
4.0	0
3.0	0

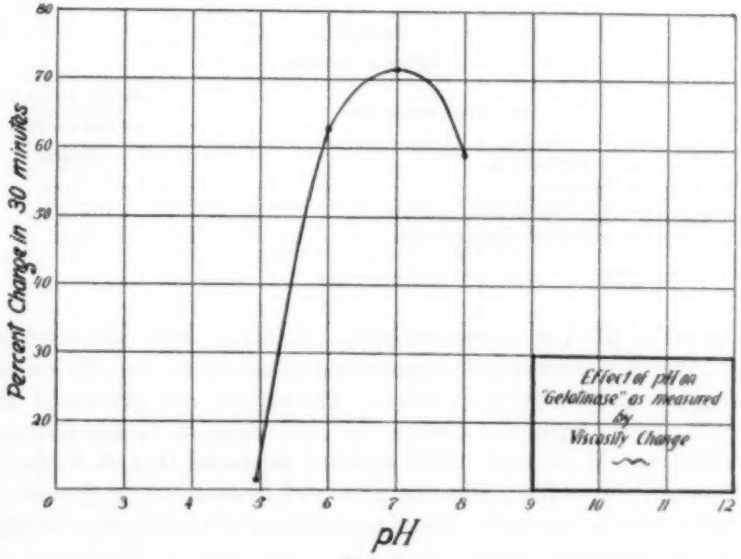

FIG. 2

cerning the effect of pH on the enzyme and on the actual viscosity change. It is quite possible that our results in this section do not represent an effect of pH on the activity of the enzyme but rather the effect of pH on the rupture of the linkages in the

protein molecule. In other words, the change in viscosity per bond hydrolyzed may be greatest at a given pH. We may have been measuring this latter effect.

Having determined the "pH of maximal activity," it remained to characterize the proteinase by activations and inactivations.

a. The effect of cysteine. Cysteine hydrochloride solution, neutralized before testing, was added to aliquots of proteinase preparations so that there were final concentrations of 0.082 M and 0.0082 M cysteine per cubic centimeter enzyme solution. The mixtures were placed in a vacuum desiccator and incubated *in vacuo* for thirty minutes. The proteinase solution was then removed and its activity against gelatin determined viscosimetrically. All components of the digestion mixture were adjusted to pH 7.0 electrometrically. Controls were composed of (*a*) suitably diluted active proteinase preparations and (*b*) boiled enzyme plus 0.082 M cysteine. The results are presented in table 3 and figure 3. Unlike the proteinase of *C. histolyticum* (Weil and Kocholaty, 1937) cysteine inhibited that of *C. botulinum*. This effect, plus the action of cyanide to be discussed subsequently, indicates that the proteinase of *C. botulinum* is not of the papainase type.

TABLE 3

Effect of cysteine

PROTEINASE PREPARATION	PER CENT CHANGE IN VISCOSITY OF GELATIN SOLUTION
(a) Untreated preparation	64.2
(b) Heat-inactivated	0.0
(c) Treated with 0.0082 Mols cysteine	64.2
(d) Treated with 0.082 Mol cysteine	52.6

Inactivation by cysteine = 11.6 per cent.

b. The effect of sodium cyanide. Solutions of sodium cyanide in M/15 phosphate buffer, pH 7.0, were prepared so that there were 0.2 mol and 0.02 mol cyanide salt per cubic centimeter. Equal volumes of proteinase preparation and cyanide solution were incubated at 37°C. for thirty minutes, after which aliquots

were removed to gelatin solution and tested viscosimetrically. Control tubes included (*a*) heat-inactivated enzyme solution containing 0.2 mol of sodium cyanide per cubic centimeter, (*b*)

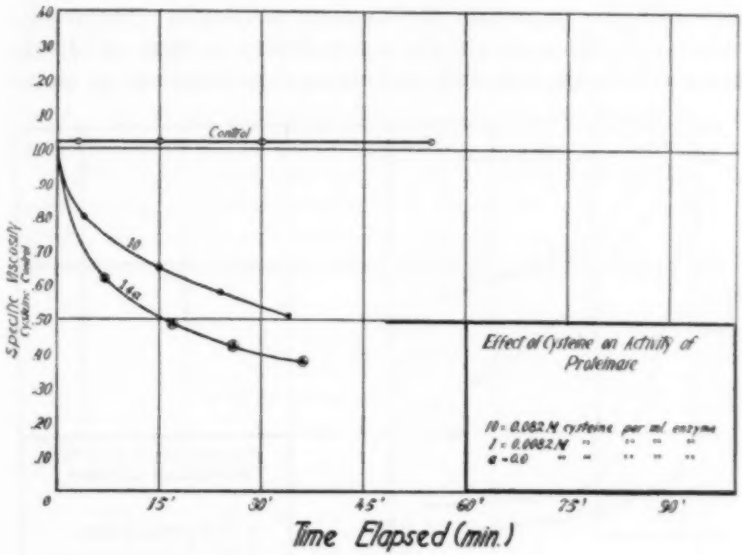

FIG. 3

TABLE 4

Effect of sodium cyanide on proteinase

PROTEINASE PREPARATION	PER CENT VISCOSITY CHANGE
(a) Untreated	45.3
(b) Heat Inactivated plus 0.1 Mol cyanide	0.0
(c) Heat Inactivated	0.0
(d) Treated with 0.1 Mol cyanide per cc. proteinase	0.0
(e) Treated with 0.01 Mol cyanide per cc. proteinase	45.3

Inactivation by 0.1 Mol sodium cyanide = 100 per cent.

heat-inactivated enzyme suitably diluted with distilled water, and (*c*) untreated enzyme suitably diluted. All pH values were checked before the mixtures were placed in the viscosimeter.

A total inhibition of the proteinase preparation by 0.1 mol sodium cyanide was observed, at pH 7.0.

Altering the pH to 5 and 9 during the cyanide inactivation did not change the effect, as Lawrie (1937) had observed to be the case with the proteinase of *Glaucoma piriformis*. The results obtained in this work are also contradictory to those of Maschmann (1937) who based his proteinase determinations on amino

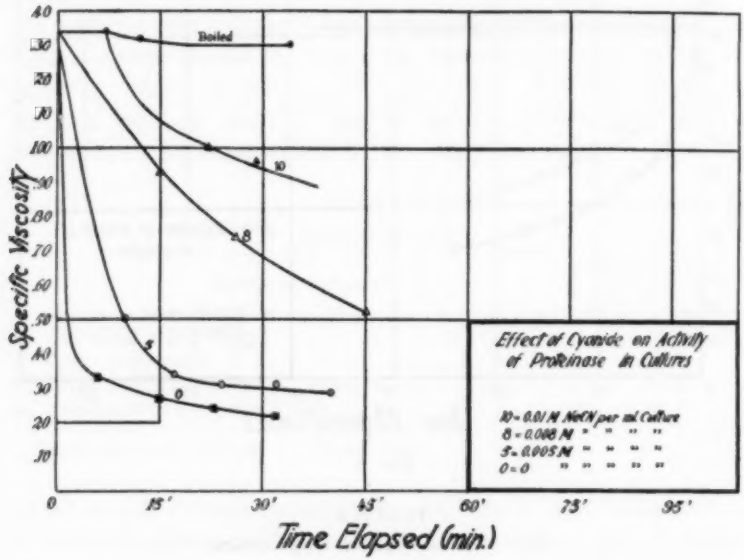
Specific Viscosity
Time Elapsed (min.)
Boiled
10
8
5
0
Effect of Cyanide on Activity of Proteinase in Cultures
10 = 0.01 M NaCN per ml Culture
8 = 0.008 M " " " "
5 = 0.005 M " " " "
0 = 0 " " " "

FIG. 4

group titrations. He found that M/100 hydrogen cyanide and M/250 cysteine activated the proteinase of *C. botulinum*.

Copper sulfate in the amount of 6×10^{-5} mol per cubic centimeter of proteinase also showed a complete inactivation viscosimetrically, both in air and *in vacuo*, in this series of experiments. The *in vacuo* tests were carried out to check the possible action of copper in catalyzing the uptake of oxygen or the oxidation of the proteinase.

It must be observed that the fundamental difference in the

methods which were used in these experiments and by Maschmann to determine "proteinase activity" may well be the cause for the divergence of results. The viscosimetric method is one predominantly involving a physical change in the micelle structure of the protein substrate. It certainly has been proven that amino nitrogen or carboxyl group titrations measure the entire proteolytic activity and not the action of the proteinase alone. Hence the seemingly contradictory results on inactivation phenomena are not strictly comparable.

c. The effect of hydrogen peroxide. To determine whether the active group on the proteinase of *C. botulinum* was of sulfhydryl nature, the action of hydrogen peroxide was tested. It was conceivable that the peroxide might oxidize such a group which

TABLE 5

Effect of hydrogen peroxide

PROTEINASE PREPARATION	PER CENT VISCOSITY CHANGE
Untreated, dialyzed	45
Heat Inactivated, dialyzed	0.0
Treated with 0.1 cc. peroxide	34.3
Treated with 1.0 cc. peroxide	31.2

Inactivation by peroxide = 13.8 per cent.

could be reactivated by the addition of hydrogen sulfide to reduce the action.

Five-cubic-centimeter aliquots of a 30-hour-old supernatant fluid at pH 7.0 received 0.1 and 1.0 cubic centimeter amounts of a 30 per cent hydrogen peroxide solution. The mixtures were incubated one hour at 37°C. and then dialyzed for one hour against distilled water in cellophane tubing to remove the peroxide. One-cubic-centimeter amounts were tested on gelatin solution. Control tubes checked the effect of (*a*) dialysis on the enzyme and (*b*) boiled dialyzed proteinase.

Attempts to reactivate the peroxide-treated proteinase solution by hydrogen sulfide and thioglycollic acid were made but were not successful. It appeared that the peroxide exerted an irreversible inactivating effect.

The ability to decrease the viscosity of gelatin solutions was paralleled by similar alterations in casein solution. However, in this respect quantitative differences were noted, namely in the amount of proteinase solution required to cause such a change. It was found that the concentration of the enzyme, considered as per cent of the total volume of the digestion mixture, had to be raised to 40 per cent for casein digestion, whereas with gelatin it amounted to 16 per cent.

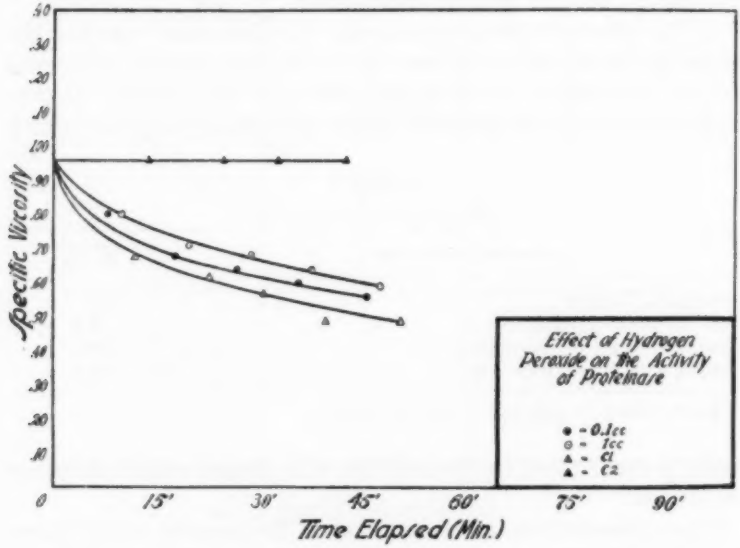

FIG. 5

The curves obtained with casein also lack the large initial change in per cent viscosity (0–15 minutes) so characteristic of gelatin digestion. A typical determination with casein is presented.

Three cubic centimeters of a 5 per cent casein solution at pH 7.0 plus 2 cc. of a proteinase preparation were tested in the usual manner. The control was composed of a boiled proteinase preparation.

It will be seen from the curves that the proteinase was able

to accomplish a 46.2 per cent change in the viscosity of the casein within thirty minutes.

Experiments on pH-activity relations, cysteine, cyanide and peroxide effects were carried out leading to the same results obtained with gelatin.

Edestin was also acted upon by the proteinase of *C. botulinum*. However, the results with this protein were not as clear cut as with gelatin or casein owing to the relative insolubility of the protein at the pH of optimum activity. Minor variations in the pH of the digestion mixture frequently caused minute flakes of edestin to form in the viscosimeter. As a result, most of the experiments on edestin had to be carried out around pH 8.0. The data at this point were however comparable to those for gelatin at the same pH.

To determine the extent of digestion of the protein of which the proteinase was capable, it was decided to use Willstätter and Waldschmidt-Leitz's alcoholic titration of the amino group (1921). Although such a method does not estimate proteinase alone, but the total activity of all the proteolytic enzymes in the fluid, it was felt at this time that preliminary experiments on whole proteins would pave the way in standardizing this procedure for the determinations of the lower peptidases.

There are approximately three typical methods for estimating amino nitrogen. These are (*a*) the nitrous acid method of Van Slyke (*b*) the formaldehyde method, and (*c*) the alcoholimetric titration. None is entirely satisfactory. The Van Slyke nitrous acid method is tedious, fortuitous errors arise from frothing and reducing agents in solution, and it gives faulty values for several amino acids and peptides. [Richardson, 1934.]

The formaldehyde and alcohol titrations are rapid and were specifically developed for enzyme and protein chemistry. Furthermore, with a glass electrode available the end points are not difficult to attain, although they are on the alkaline side in a range where the electrode works with less precision. With control solutions treated identically, the values are only used in relation to each other and approximately the same electrode

error obtains in both the test and control series. The error of the electrode in this region (10.5) is equalled by the error in the colorimetric method.

TABLE 6

Alkalimetric determination of pH-activity curve

pH OF PROTEINASE PREPARATION	N/5 KOH	PER CENT CHANGE (BASED ON CHANGE AT pH 7.15)
	cc.	
5.0	0.2	36.3
6.0	0.32	58.1
7.15	0.55	(100.) (arbitrary)
8.0	0.23	41.8
9.15	0.19	34.5

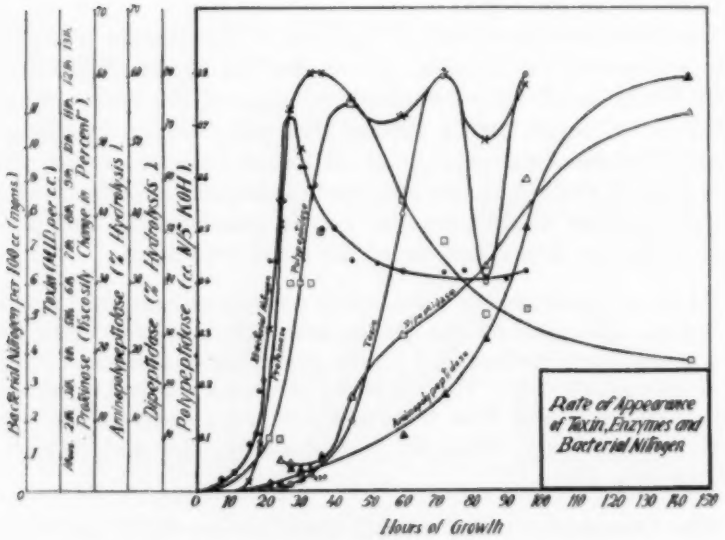
Bacterial Nitrogen per 100 cc (mgms.)
Toxin (MLD per c.c.)
Proteinase (Viscosity Change in Percent)
Aminopolypeptidase (% Hydrolysis)
Dipeptidase (% Hydrolysis)
Polypeptidase (cc N/5 KOH)
Hours of Growth
Rate of Appearance of Toxin, Enzymes and Bacterial Nitrogen

FIG. 6

The presence of phosphate buffer salts in the medium which interfered with the formol titration, plus the possible stepwise procedure in the alcoholic method, made it more profitable to use the latter method.

For completion the following experiment, relating activity to pH range, is presented.

Fifteen cubic centimeters of gelatin solution adjusted electrometrically with hydrochloric acid or sodium hydroxide to the desired pH were mixed with 3 cc. of supernatant fluid, also adjusted to proper pH value, and incubated 18 hours at 37°C. Controls consisted of boiled proteinase preparations or, in other cases, aliquots of the test mixture titrated immediately on mixing. The pH was adjusted to 7.0, and absolute alcohol added until the final alcohol concentration was 90 per cent. The mixtures were then titrated with N/5 KOH in 90 per cent alcohol to pH 10.5 by means of the glass electrode.

Comparison between the shape of the curve obtained by alkalimetric determination and that obtained viscosimetrically reveals an identity of optimum points but also shows in the former case a more gradual loss of activity on the alkaline side.

THE POLYPEPTIDASES

a. "Polypeptidase" activity was determined by using a 1 per cent Witte's peptone solution, at pH 7.4. Eight cubic centimeters of the peptone solution were mixed with 4 cc. of the supernatant fluid, and a 5 cc. aliquot withdrawn immediately for the control titration. The remainder of the digestion mixture was incubated at 37°C. and a 5 cc. aliquot titrated.

b. Aminopolypeptidase activity was determined with d,-l-leucylglycyl-glycine. A M/10 solution was prepared and adjusted to pH 7.8; 4 cc., containing 0.0004 mol, were mixed with 2 cc. supernatant fluid. A 3 cc. aliquot was removed immediately for the control titration and the remainder incubated at 37°C. for 18 hours.

c. Carboxypolypeptidase activity was determined with chloracetyl-l-tyrosine as substrate. A M/25 solution was used, of which 10 cc., containing 0.0004 mol, at pH 7.8, was mixed with 4 cc. supernatant fluid. An aliquot of 7 cc. was removed immediately for the control titration and the rest was incubated 18 hours at 37°C.

d. Dipeptidase was determined with d,l-leucyl-glycine and

glycyl-glycine as substrates. M/10 solutions were prepared, of which 4 cc., containing 0.0004 mol, at pH 8.0, were mixed with 2 cc. supernatant fluid. A 3 cc. aliquot was removed immediately for the control titration and the remainder titrated after 18 hours at 37°C.

e. Tri-glycine splitting ability was determined with diglycyl-glycine, using M/10 solution, at pH 7.8. The digestion mixture contained 4 cc. (0.0004 mol) substrate plus 2 cc. supernatant fluid. The usual control was titrated and the test incubated 18 hours at 37°C.

It was to be expected from the growth of *C. botulinum* in commercial peptones that a "polypeptidase" would be present in the

TABLE 7

Peptidase activity of a 48-hour-culture supernatant fluid

ENZYME DETERMINED	TITRATION VALUE (CC. N/5 KOH)	PER CENT OF THEORETICAL HYDROLYSIS
1. "Polypeptidase" (48 hrs.)	0	0
(24 hour culture)	0.8	
(72 hour culture)	0.25	
2. Aminopolypeptidase	0.03	3.0
3. Carboxypolypeptidase	0.0	0
4. Dipeptidase:		
(a) Leucyl glycine	0.06	6
(b) Glycyl glycine	0.0	0
5. Tripeptidase (Diglycyl glycine)	0.0	0

extracellular system. The significance of this hydrolysis is of course not clear, owing to the uncertainty of the chemical nature of the substrate, and to the fact that proteinases also attack peptones. The ability of the supernatant to decompose "Proteose Peptone" would indicate the ability of the organism to produce peptidases acting on polypeptides of more than three amino acids in the chain.

The lack of ability to decompose chloracetyl-l-tyrosine, indicating the absence of a carboxypolypeptidase, was confirmed by testing supernatant fluids every 24 hours over a period of 240 hours at pH values from 4 to 10. At no time was there any indication of such an enzyme in the extracellular system.

The presence of an aminopolypeptidase was confirmed repeatedly. It was also determined that after 5 to 6 days the hydrolysis would exceed 50 per cent, indicating that both optically active forms were hydrolysed. This enzyme in supernatant fluids of *C. botulinum* was also found by Maschmann and he observed that it was specifically inactivated by cysteine and cyanide.

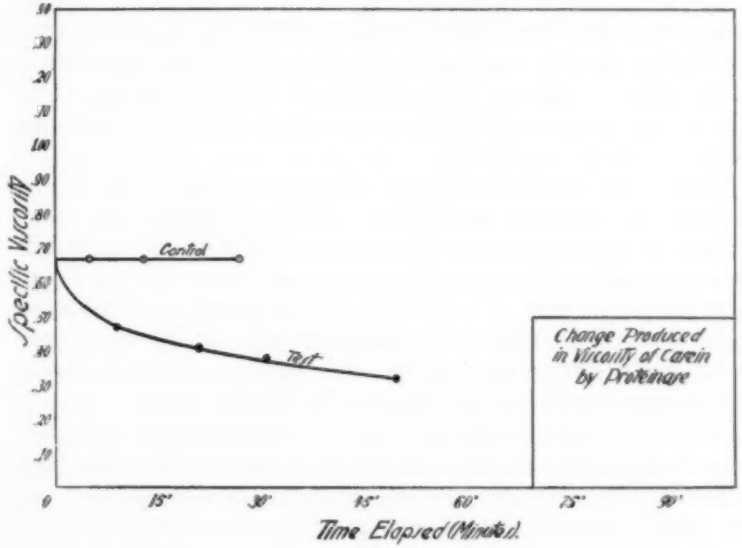

FIG. 7

The presence of a dipeptidase was confirmed, and in the case of *Clostridium parabotulinum*, it was also found that peptides containing only molecules of glycine were not hydrolysed. Johnson and Peterson (1935 a, b) noted this same specificity in the case of the dipeptidase of *Aspergillus parasiticus*. It was observed that, in the case of *C. botulinum*, apparently both optically active forms were hydrolysed inasmuch as the per cent hydrolysis was often equivalent to more than 50 per cent.

The inability to hydrolyse diglycyl glycine further emphasized the specificity of the polypeptidases.

The optimum points for activity of the aminopolypeptidase and dipeptidase in the extracellular system were determined to be pH 7.8 and 8.0 respectively.

It was decided to postpone indefinitely further experiments on activation and inactivation procedures on these enzymes until a time-correlation between them and toxin production could be tentatively established.

The extracellular proteolytic system of *C. parabotulinum*, A, apparently consists at least of a "proteinase," which changes the viscosity of gelatin, casein and edestin, and aminopolypeptidase, a dipeptidase, and a "Polypeptidase." In this system there are no carboxypolypeptidases nor are there enzymes hydrolyzing chains of glycine molecules.

In the case of *C. botulinum* certain experiments have been reported of potential significance. Stark, Sherman and Stark (1928a, b) allowed sterile filtrates of cultures to act on sterile skimmed milk, purified casein, and yeast cells. It was found that when one part of toxic filtrate was mixed and incubated with 49 parts of milk a seven-fold increase in the M.L.D. per cubic centimeter content of the toxic mixture resulted. These results were confirmed and extended by Meyer (1928).

The apparent significance of the extracellular enzymes, plus the fact that no direct approach to this problem has been reported, where the enzymes have been materially inhibited and the toxin formation determined, gave impetus to the collection and presentation of relevant data. An examination of the proteolytic enzymes present in the surrounding medium during all phases of growth has been attempted.

Experimental

The first step taken was to determine the time of appearance of toxin and the extracellular proteolytic enzymes.

Experiment 1. Tubes containing 10 cc. of glucose-beef heart medium prepared as described in the introduction were inoculated with a suspension of dried spores. The inoculum was small enough so that no change in turbidity of the medium resulted. At stated periods tubes were removed from the series, centrifuged

to remove the organisms, and the latter removed and washed in saline. Before the final washing and centrifugalization, the organisms were transferred to conical micro-Kjeldahl centrifuge flasks. The sediment was then resuspended in 1 cc. distilled water.

The Kjeldahl method for determining nitrogen, modified by Pregl (1930), was used to determine the bacterial nitrogen. One cubic centimeter of concentrated sulfuric acid was added to the suspension, which also received a knife-point of a mixture of copper and potassium sulfate (1:2). It was also found advisable to accept Mueller's recommendation (1935) that 4 drops of 30 per cent hydrogen peroxide be added three times during the digestion. The distillations were carried out in an all-glass apparatus designed and made by Mr. Cummings of the University Chemistry Department.

On the supernatant fluid from the centrifuged cultures determinations of proteinase, aminopolypeptidase, dipeptidase, and "polypeptidase" were made.

Another aliquot of the same supernatant fluid was diluted in a series of 10-fold dilutions and the toxin concentration determined in terms of M.L.D. per cubic centimeter by mouse inoculation. Duplicate mice were used for each dilution. Five-tenths cubic centimeter of each dilution was inoculated intraperitoneally and the amount required to kill a 20 gram mouse in 48 hours determined as closely as possible.

The composite data for a typical run are presented in table 8.

It will be observed that maximum bacterial nitrogen is attained around the 27th hour, after which a slow decline occurs, accompanied by a visible decrease in turbidity of the culture fluid. The nitrogen values level off and remain fairly constant thereafter.

Toxin begins to appear as early as the 21st hour of growth, reaching a peak long after the bacterial nitrogen maximum, and, it will be noticed, during the phase of visible autolysis.

The proteinase parallels the bacterial nitrogen curve, and appears about 6 hours before toxin is detectable. On the other hand the lower peptidases do not appear in appreciable amount until long after the toxin has appeared. One would be led to

believe that, if any of the enzymes might bear a causal relationship the proteinase would bear further investigation.

Experiment 2. Sodium cyanide solutions were prepared in M/15 phosphate buffer at pH 7.0. Ten-cubic-centimeter tubes of glucose-beef heart medium were adjusted to contain varying

TABLE 8

GROWTH	BACTERIAL NITROGEN PER 100 CC.	TOXIN (M.L.D. PER CC.)	PROTEINASE (VISCOSITY CHANGE)	AMINOPOLYPEPTIDASE	DIPEPTIDASE	POLYPEPTIDASE (CC. N/5 KOH)
hours	*mgm.*		*per cent*	*(per cent hydrolysis)*	*(per cent hydrolysis)*	
7	0.33	0	0	0	0	0
	0.50					
15	1.35	0	1.3	0	0	0
	1.68					
18	2.94	0	6.9	0	0	0
	3.20					
21	6.65	20	23.4	1	0	0.1
	6.65					
24	8.40	80	38.6	1	6	0.1
	8.39					
27	10.8	160	55.1	3	5	0.4
30	9.38	400	49.4	3	3	0.4
	9.38					
33	8.82	480	60.5	3	3	0.4
	8.86					
36	7.56	800	60.5	5	6	0.5
	7.59					
45	6.65	2,000	57		18	0.75
	6.58					
60	6.33	8,000	54.1	8	30	0.56
72	6.30	12,000	60	14		0.48
	6.37					
84	6.16	6,000	50.5	22	43	0.32
	6.11					
96	6.37	12,000	58.8	38	60	0.35
144				60	73	0.25

amounts of the cyanide salt and were then inoculated with a heated spore suspension. Sets of ten tubes per concentration of cyanide were used.

In 30 hours, tubes containing 0.01 mol, and less, cyanide per cubic centimeter medium showed growth equal to that in the

control tubes containing no cyanide. Tubes containing more than 0.01 mol sodium cyanide per cubic centimeter medium eventually showed growth but the appearance of this growth was sometimes delayed for 8–10 days. 0.02 mol was the upper limit of cyanide concentration (per cubic centimeter medium), above which no growth ever occurred.

Only tubes showing growth as soon as the control series (with no cyanide) were tested inasmuch as it was desired to simulate natural conditions as much as possible.

The proteinase tests were carried out in the usual manner with gelatin solution, at pH 7.0.

TABLE 9

Effect of sodium cyanide on production of proteinase

PROTEINASE PREPARATION	PER CENT CHANGE IN VISCOSITY
1. Untreated culture	83.5
2. Secreted in presence of 0.005 Mol per cc	77.5
3. Secreted in presence of .008 Mol per cc	52.3
4. Secreted in presence of 0.01 Mol per cc	28.3
5. Heat-inactivated	0.0
"Inactivation" by sodium cyanide in medium:	
0.005 Mol	7.2 per cent
0.008 Mol	38.3 per cent
0.01 Mol	66.2 per cent

The toxin determinations were made for M.L.D. per cubic centimeter of medium. Inasmuch as the tubes with 0.005, 0.008 and 0.01 mol sodium cyanide allowed growth at a rate equal to that of the control tubes, they were chosen for assay. The proteinase tests are presented in table 9.

That the proteinase was not simply altered in its rate of appearance rather than in the total amount produced was tested by examining over a period of 96 hours the proteinase content of the medium. The maximal point was reached in every case around the 30th hour with no further increase.

If the extracellular proteinase is directly concerned with toxin production, then tubes containing 0.008 and 0.01 mol. sodium

cyanide per cubic centimeter of medium should show a decreased amount of toxin, around the 96th hour when it is usually maximum in this medium. Such did not prove to be the case, however, as the data in table 10 indicate.

Tubes in which the proteinase showed 60 per cent inhibition or reduction in concentration were inoculated in 10-fold dilutions into mice in the usual manner. Control mice received antitoxin to check the possible effect of the cyanide and heat-inactivated toxin to further check the natures of the killing agent. A third series of mice received the supernatant fluid from cultures grown in the absence of cyanide.

The complete similarly in picture between the cultures grown in the presence and absence of sodium cyanide was confirmed

TABLE 10

TOXIN DILUTION (0.5 cc.)	CYANIDE-TREATED CULTURES	UNTREATED CULTURES	"HEAT-KILLED" TOXIN	ANTITOXIN-PROTECTED
10^{-2}	Dead—18 hours	Dead—18 hours	No symptoms	No symptoms
10^{-3}	Dead—26 hours	Dead—28 hours	No symptoms	No symptoms
10^{-4}	Dead—45 hours	Dead—43 hours	No symptoms	No symptoms
10^{-5}	Dead—55 hours	Dead—50 hours	No symptoms	No symptoms
10^{-6}	Symptoms severe	Symptoms mild	No symptoms	No symptoms
10^{-7}	No symptoms	No symptoms	No symptoms	No symptoms

on several repetitions. It was therefore concluded that a 60 per cent reduction in the proteinase activity of the extracellular system does not cause a reduction in the toxin concentration detectable in this manner. It remains to test this assumption again on very much larger numbers of mice for statistical evaluation.

Experiment 3. The fact that the efficiency of the bacterial enzyme systems may exert a masking influence, and that the 40 per cent remaining activity of the enzyme may still account for toxin production was tested by examining the distribution of the proteinase in types of *C. botulinum.*

Four "B" types, two "A" types, a "C" and a "D" types were examined. The data are presented in table 11.

For comparison, strains 526 C, 237 B, D, and 63 B were selected

for toxin determinations. Titrations of the supernatant fluid from 96-hour-old cultures in mice again failed to show any correlation with the proteinase activity as the data in table 12 indicate.

The cultures from Type D and strain 237 B show on one hand maximal deviation in enzyme activity and on the other a quantitative similarity in toxin-production ability. Strain 63 B

TABLE 11

Proteinase distribution

STRAIN	PER CENT CHANGE IN VISCOSITY IN 30 MINUTES BY 1 CC. CULTURE FLUID	
	24 hour culture	48 hour culture
237 (B)	83.6	81.0
34 (B)	74.5	39.3
London (A)	63.9	73.4
63 (B)	59.0	58.3
69 (A)	54.0	
6 (B)	30.3	43.9
526 (C)	9.8	0.0
(D)	0	0.0

TABLE 12

Distribution data of proteinase and toxin

STRAIN	PROTEINASE ACTIVITY	M.L.D. PER CC.
237	83.6	200,000
526	9.8	20,000
D	0	200,000
63	59	20,000

showing a moderately strong proteinase belongs in the same class toxigenically speaking with 526 C, which is practically inert as far as protein-splitting ability is concerned.

It would appear therefore very probable that the residual activity of the extracellular proteinase in cyanide-treated cultures was not a factor in toxin production but more probably concerned the maintenance of the bacterial "*status quo*."

The question concerning the designation of a toxin as an exo-

toxin or an endotoxin and the implications arising from such terms have recently been brought to attention once more.

The neurotoxins of *Corynebacterium diphtheriae*, *Clostridium tetani* and *Clostridium botulinum* have been generally accepted as classical examples of exotoxins. It is certainly true, in addition, that these toxins possess characteristics which at once distinguish them from the endotoxins of the cholera vibrio and the meningococcus. However, the true state of affairs in designating these toxins as endotoxins or exotoxins has been aptly summarized by Topley (1933) in the statement that "although this broad distinction can be made by comparing typical endotoxins with typical exotoxins, our difficulties become great if we attempt to assign each bacterial product to its correct group. . . ."

The data on the time of appearance of diphtheria, tetanus and botulinus toxins show that these toxins do not appear until the cells have reached the maximum growth phase, and, in fact, as our results show, botulinus toxin appears maximally during the phase when most of the cells are autolyzing.

The results of Eaton (1936a, b, 1937) have shown almost conclusively that the diphtheria toxin is a protein, which is heat-coagulable and which can be denatured by acids. Pappenheimer (1937a, b, c) has studied the purification of diphtheria toxin in a far simpler medium and also believes that the diphtheria toxin is a protein. Both investigators have isolated this toxic protein from media in which no protein tests were given before inoculation. The period of incubation before the toxin was harvested was around five to six days, enough time to elapse for considerable lysis of cells if reference is made to a paper by Moloney and Hanna (1921) and to the more recent and accurate studies of Mueller (1935).

The evidence indicates that diphtheria toxin is formed inside of the cells and is set free on the death and destruction of the cells.

Our results with botulinus toxin also point to its intracellular formation on the basis of the rise in M.L.D. when visible autolysis occurs. Furthermore, the difficulty of obtaining atoxic sus-

pensions of diphtheria and botulinus bacilli by washing is well-known. With diphtheria it is possible to produce anti-bacterial sera which are not antitoxic, as Eaton (1936b) succeeded in demonstrating. However, in the case of botulinus, Thom, Edmondson and Giltner (1919), Burke, Elder and Pischel (1921) and Nelson (1927) have stated that it is impossible to prepare botulinus bacilli or spores suspensions free of toxin, even after fifteen to twenty washings. In fact, Nelson (1927) found that a mass of botulinus bacilli could be extremely toxic despite the atoxicity of the broth fluid in which they had grown.

It would appear therefore that, with *C. botulinum*, one is dealing with an endotoxin, or at least with a toxin the locus of production of which is inside the cell. This change in conception does not in fact complicate the toxin make-up of bacteria because Boivin, Mesrobeaunu, Topley *et al* have shown that the classical endotoxins are very closely related, if not identical with, the somatic antigen. The toxin production of bacteria can therefore be pictured as occurring entirely within the cell. The true non-antigenic endotoxins may be considered intimately bound or identical with the soma whereas the characteristic antitoxinogenic toxins, also produced intracellularly, may be considered as substances to which the cell membrane is impermeable during the life of the cell, and which are distinct from the somatic antigens of the cell.

SUMMARY

1. The extracellular proteolytic system in relation to toxin production was studied. It was found that a "proteinase," acting on gelatin and casein was secreted into the medium. This enzyme acted optimally at pH 7.0. It was inactivated by sodium cyanide, cysteine, hydrogen peroxide and copper salts.

2. The lower members in the proteolytic system included a polypeptidase acting on Witte's peptone, an aminopolypeptidase, hydrolyzing leucyl-glycyl glycine and a dipeptidase, hydrolyzing leucyl glycine. The optimal pH values for these enzymes was 7.8–8.0.

3. Experiments on rate of appearance revealed that the

proteinase appeared first in the culture fluid, paralleling the trend of bacterial nitrogen. It also appeared at least six hours before the toxin could be demonstrated.

4. The polypeptidases and dipeptidase appeared simultaneously with toxin but, whereas the toxin reached its maximum around the ninety-sixth hour, these enzymes slowly increased during the first six days.

5. When, during the normal growth of the organisms, the proteinase was inactivated by the presence of sodium cyanide in the medium, the production of toxin was unchanged and unaltered.

6. Various types of *Clostridium botulinum* showed no correlation between the extracellular proteolytic enzymes and toxin production. Types "C" and "D" produced large amounts of toxin, comparable with the proteolytic type "A" in the absence of any detectable quantities of proteinase. Strains of type "B" on the other hand produced amounts of proteinase comparable to that of type "A" but produced approximately one-tenth as much toxin.

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METABOLIC STUDIES OF A NON-HEMOLYTIC STREPTOCOCCUS[1]

J. W. KING, J. C. GAREY, AND M. A. FARRELL

Division of Bacteriology, The Pennsylvania State College, Pennsylvania

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INTRODUCTION

The nutrition of the non-hemolytic streptococci, which occur often as human and animal pathogens, has been studied relatively little. A member of this group of streptococci was selected because of earlier studies using this organism, and also because it was felt that information obtained with this culture might aid in the solution of the nutritional requirements of the more fastidious hemolytic streptococci.

Earlier workers on the metabolism of streptococci have confined themselves almost wholly to hemolytic strains. Kobayshi and Nishikawa (1921) reported that a hemolytic streptococcus studied by them responded to the addition of .005–.5 per cent cystine and .02–.2 per cent histidine hydrochloride to sheep-serum media.

Gordon and McLeod (1921) studied the effect of amino acids on a number of organisms, including several hemolytic streptococci. They reported high concentrations of amino acids to be very readily inhibitory to the growth of these bacteria.

Mueller (1922a) showed that casein acid digests and beef infusions contained a growth factor for hemolytic streptococci. He also (1922b) showed that the factor could be removed by absorption of the digests or infusions on charcoal. Tryptophane, tyrosine, cystine, and histidine would not substitute for this factor.

Freedman and Funk (1922a-b), using a hemolytic streptococcus as a test organism, also observed this growth factor in a number of substances, including beef-'muscle' and beef-heart infusions, peptone and brewer's yeasts.

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Whitehead (1924) also reported the presence of a growth factor for hemolytic streptococci in the Dakin butyl-alcohol-insoluble fraction of casein acid digests. He studied various amino acids and reported (1926) that inorganic phosphates were probably more important than amino acids in the nutrition of streptococci.

Thompson (1929) observed the effect of vegetable and beef heart extracts on the growth of hemolytic streptococci which were not able to grow on nutrient broth. The growth-promoting power of the extracts was destroyed by heat.

Krasnow and Rosenberg (1929) and Krasnow and Gies (1929) studied synthetic media, supplemented with such simple sources of nitrogen as amino acids and ammonium salts. In the 671 and 441 media studied respectively, none gave growth past the second subculture, with the strains of dental streptococci used.

Farrell and Thomas (1932) using a gamma-type streptococcus called *Streptococcus rheumaticus* observed survival times on various synthetic media, using ammonium salts, amino acids, and amines as the nitrogen sources. In general, they found that phenylalanine, leucine, cystine, glutamic acid and histidine were beneficial to the survival of the culture.

Koser and Saunders (1935) isolated a substance from beef extract which stimulated the growth of streptococci and other bacteria. Very small amounts of this factor, when added to a synthetic base, fostered growth.

Smith (1938a-b) observed that the production of haemolysin by hemolytic streptococci was strongly affected by changing the concentration of peptone in the medium. Excess peptone, especially the dialysable fraction, inhibited the production of haemolysin.

Hutner (1938) has studied the effect of various fractions of casein acid digests obtained by precipitation and by other methods on the growth of the hemolytic streptococci. He showed that the sulfur-bearing amino acids were very important in streptococcal metabolism.

Koser, Finkle, Dorfman, Gordon and Saunders (1938) attempted to cultivate a hemolytic streptococcus in a synthetic medium of salts, amino acids, etc. Various factors were added, such as tissue extracts, the sporogenes vitamin and the growth

factor for the propionic acid bacteria. No growth was observed with any of these substances.

It was the purpose of the present study to investigate the growth-promoting ability of casein acid hydrolysates and the fractions of such hydrolysates for a representative pathogenic, non-hemolytic streptococcus. In addition, upon learning which fraction or fractions stimulated growth the amino acids known to be present in such fractions were studied individually to determine which of these substances is most essential to the nitrogen metabolism of this streptococcus.

PROCEDURE

The casein acid hydrolysates used in this study were prepared and fractionated according to the method of Dakin (1918). Briefly, this consisted of digesting a technical grade of casein in a refluxing condenser with 30.0 per cent sulphuric acid. The sulphuric acid was removed quantitatively with barium hydroxide after diluting the digest to several volumes with distilled water. The neutralized digest was concentrated *in vacuo* to a volume of about 500 cc. per 100 grams casein used. The digest thus prepared was allowed to stand in the refrigerator over night. The more insoluble tyrosine and part of the leucines were precipitated and removed by filtering. The remaining digest was made up to 500 cc. volume per 100 grams casein. It was then fractionated in a Kutscher-Steudel extractor with normal butyl alcohol. The resulting three fractions, when purified according to Dakin consisted of the following groups of substances:

Fraction A: monamino, monocarboxylic amino acids, less tyrosine and a part of the leucine isomers. This fraction is extracted in butyl alcohol and precipitates out as a white powder.

Fraction B: proline and hydroxy proline which remains in solution in the butyl alcohol in the extraction flask.

Fraction C: the butyl-alcohol-insoluble fraction, made up of the diamino and dicarboxylic amino acids and the heterocyclic acids. Tryptophane is absent from this group as it is destroyed by acid digestion.

The fractions thus secured were sterilized by autoclaving at 15 pounds for 15 minutes.

The organism employed in this study, *Streptococcus rheumaticus*, Allen strain, was a representative strain of a non-hemolytic, pathogenic streptococcus which was secured from the Lister Institute. The organism was a very short-chained streptococcus; it grew well on the beef-infusion stock culture medium and poorly, or not at all, on 0.3 per cent beef-extract water. This strain had been carried in the stock culture collection on ground-meat medium. During the period of this study the culture was carried on beef-infusion agar, pH 7.4–7.6, and transferred every other day over a period of six months before being used in this investigation. This was found necessary because inconsistent results were obtained when care was not taken to assure a young, active, uniform culture. Older cultures tended to become adapted to the basal medium and in many cases made duplication of results difficult.

The basal medium, to which the hydrolysate fractions and amino acids were added, was 0.3 per cent beef extract (Difco) water. (It was presumed that the carbon and mineral sources were supplied by this extract.) In all cases where the volume was altered by adding amino acid solutions, the basal medium was so prepared as to provide the proper final volume and concentration of all constituents. The reaction of these media was pH 7.4–7.6 and all supplementing materials were checked for proper pH values. The media containing digest fractions were prepared as indicated in the several tables. When a medium is shown as containing 5.0 per cent of a fraction, it is to be interpreted that each 10 cc.[2] of culture medium contains 0.5 cc. of the fraction indicated. These media were sterilized by autoclaving at 15 pounds for 15 minutes. The amino acids used in this study were made up in solutions of varying concentrations (depending upon the solubilities of the acids), and sterilized by filtration. They were added in the required amounts, as later determined, to prepared and sterilized beef extract water.

All estimations of growth were made for final recording after culturing the organism on the medium under consideration three or four times. It was assumed that the third subculture should

[2] All media were dispensed so that each culture tube contained 10 cc. of medium.

give growth which was not affected by food materials carried over with the inoculum from the infusion-broth culture medium. Inoculations were made with a straight nichrome needle. In preliminary investigations growth was estimated by visual estimations of turbidity and by colorimetric and volumetric micro-Kjeldahl procedures. These methods were not satisfactory. The first, because it involved excessive dependence upon the human factor, could hardly be considered as more than semi-quantitative. The second and third procedures are inaccurate because they both require that the cells be separated from the culture medium. This is very difficult when dealing with small amounts of culture medium and is especially difficult with streptococcal cells of this type, which do not form a pellicle or pack well. For these reasons growth was measured by using a photoelectric colorimeter to determine the turbidity of cell suspensions.[3]

The turbidity of each culture was determined in triplicate against the uninoculated controls. The resulting readings were treated as ordinary colorimetric values and the index of growth produced was secured by dividing the reading of the culture cup into the reading of the uninoculated culture medium control. By reducing the denominator of this fraction to one, the ratio or index thus secured was then used as a value to compare with a similar value secured with the beef-extract water culture. The resulting expression:

$$\frac{\text{Index of growth of any culture medium}}{\text{Index of growth of the beef-extract water}} = \text{growth index}$$

was used as a means of comparing growth-promoting ability.[4]

[3] Since the turbidity produced by the growth of the strain used was very even it was felt that if it were possible to measure turbidity by some mechanical means a relatively satisfactory method of estimating growth could be devised.

[4] In order to make clear just how this growth index was derived an example is given:

	Colorimetric readings of uninoculated control cup	*of inoculated medium*
Medium X	21.0	10.0
Beef extract	12.0	10.0

Growth index of medium X = 21/10 = 2.10.

Growth index of beef-extract medium—12/10 = 1.20.

Growth index as recorded in final column of the table then was: 2.10/1.20 = 1.75.

A comparison of these values with micro-Kjeldahl determinations of bacterial nitrogen showed the following correlation:

Growth index	*Mgm. bacterial nitrogen per 100 cc. of culture*
2.4	73.4
2.0	66.0
1.7	54.2
1.5	33.4

These indexes are believed to be a fairly accurate representation of the growth-promoting or inhibiting ability of the substances under investigation.

TABLE 1

The effect of different concentrations of unfractionated casein hydrolysate on the growth of Streptococcus rheumaticus

DIGEST PER 100 CC. MEDIA	CONC. BEEF EXTRACT	FIRST CULTURE	SECOND CULTURE	THIRD CULTURE	GROWTH INDEX
cc.	*per cent*				
None	0.3	+*	+	+	1.00
2.5	0.3	++	++	+	1.37
5.0	0.3	++	++	+	1.22
7.5	0.3	++	++	++	1.79
10.0	0.3	++	++	++	1.79
15.0	0.3	++	++	++	1.53
20.0	0.3	++	++	++	1.71
25.0	0.3	+++	+++	++	1.44

* − = no growth, + = trace of growth, ++ = poor growth, +++ = good growth.

THE GROWTH-PROMOTING ABILITY OF CASEIN HYDROLYSATES

In order to determine the influence of casein hydrolysates on the growth of *Streptococcus rheumaticus*, media were prepared containing concentrations of this hydrolysate up to 25 cc. per 100 cc. of basal medium. The inoculation and subculture procedure used in this and the remaining experiments in this paper was as follows: The media were inoculated from a 48-hour infusion agar culture of the test organism. After 48 hours incubation at 37°, subcultures were made into the respective media used. A third subculture was made at the end of another 48 hours incubation

from the second culture. All three cultures were examined for growth by visual estimation of turbidity after 48 hours incubation, and the third culture was also examined by means of the photoelectric colorimeter. The results are shown in table 1.

Table 1 shows that there is a marked stimulation in the growth of *Streptococcus rheumaticus* when reasonably small amounts of casein hydrolysates are added to the basal medium. While the best growth occurred in basal media containing 7.5 and 10.0 per cent of the hydrolysate, the values on either side of these two percentages are not great. Other hydrolysate preparations showed a higher growth index when 5.0 per cent was employed and for other studies in this paper it was decided arbitrarily to use the five cubic centimeter portion of the whole hydrolysate per hundred cubic centimeters of basal medium.

THE GROWTH-PROMOTING PROPERTIES OF THE THREE FRACTIONS OF THE CASEIN ACID HYDROLYSATE

In order to determine the influence of the individual fractions prepared by butyl-alcohol extraction on the growth of this streptococcus, 0.3 per cent beef-extract water media were prepared, supplemented with the individual fractions, alone and in combination with each other. The results are shown in table 2.

Table 2 shows the influence of the three fractions of the casein acid digest on the growth of *Streptococcus rheumaticus*. From the data presented it is evident that the greatest growth stimulation occurrs when fraction C is used either alone or in combination with fraction A. Fraction A alone stimulates growth of this streptococcus but slightly, while fraction B either alone or in combination with either or both of the other fractions inhibits growth. While the use of fractions A and C gives almost as much growth as the same per cent of the unfractionated hydrolysate, the use of all three fractions gives but a very slight growth stimulation. The inhibitory substances present in the B fraction may account for the difference.

Since the C fraction, containing diamino and dicarboxylic amino acids, was the portion of the hydrolysate showing the greatest growth stimulation, it was decided to determine the

influence of the concentration of this fraction on the growth of this streptococcus. Table 3 shows the results of inoculating media prepared with increasing amounts of fraction C.[5]

TABLE 2

The effect of the various hydrolysate fractions on the growth of Streptococcus rheumaticus

CONC. FRACTION A	CONC. FRACTION B	CONC. FRACTION C	FIRST SUBCULTURE	SECOND SUBCULTURE	THIRD SUBCULTURE	FOURTH SUBCULTURE	GROWTH INDEX
cc./100 cc.	*cc./100 cc.*	*cc./100 cc.*					
None			−	+	+	+	1.00
5.0			±	+	+	+	1.23
	5.0		−	−	−	−	0.99
		5.0	++	++	+	++	1.59
5.0	5.0		−	−	−	−	1.00
5.0		5.0	++	++	++	+++	1.83
	5.0	5.0	−	−	−	−	1.01
5.0	5.0	5.0	−	−	−	−	1.05
5.0*			++	++	+++	+++	1.85

* cc. of the unfractionated hydrolysate.

TABLE 3

The effect of different concentrations of fraction C on the growth of the test organism

CC. OF FRACTION C PER 100 CC. OF MEDIUM	BEEF EXTRACT	FIRST SUBCULTURE	SECOND SUBCULTURE	THIRD SUBCULTURE	GROWTH INDEX
	per cent				
None	0.3	−	+	±	1.00
3.0	0.3	+	+	+	Lost
5.0	0.3	+	+	+	1.23
10.0	0.3	+	+	++	1.26
15.0	0.3	+	+	++	1.21
20.0	0.3	+	+	±	1.52
30.0	0.3	+	+	±	1.38

The data in table 3 show that under the conditions of this study 20 cc. of digest per 100 cc. of medium gives the greatest amount of growth. Since we were not interested in the maximum

[5] As a check to see whether concentration of Fraction A would affect its growth promoting powers, a series similar to this C series was prepared with the A fraction. The results showed little, if any, stimulation of growth with the A fraction.

cell growth but rather in a plentiful supply of the growth factors under consideration 5.0 per cent of the C fraction was used in subsequent studies.

GROWTH-INHIBITORY PROPERTIES OF THE B FRACTION

Previous experiments showed that the B fraction inhibits the growth of the test organism. This property was present in most, but not all, of the digests prepared and studied. As it was never present in the hydrolysate before butyl-alcohol extraction, it must have arisen as the result of the fractionating process or the subsequent purification of the fraction from the butyl alcohol.

TABLE 4

The effect of various concentrations of fraction B on the growth of Streptococcus rheumaticus

CONC. OF B FRACTION	CONC. A AND C FRACTIONS	BEEF EXTRACT	FIRST SUB-CULTURE	SECOND SUB-CULTURE	THIRD SUB-CULTURE	FOURTH SUB-CULTURE	GROWTH INDEX
	cc./100 cc.	*per cent*					
None	5.0	0.3	++	++	++	++	1.00
0.25	5.0	0.3	+	++	+	+	0.89
0.50	5.0	0.3	+	++	+	+	0.89
1.00	5.0	0.3	−	−	±	±	0.92
2.00	5.0	0.3	−	−	+	−	0.46
3.00	5.0	0.3	−	−	+	−	0.50

In studying the influence of the B fraction on the growth of this streptococcus, various percentages of this fraction were added to a basal medium consisting of the usual 0.3 per cent beef extract water containing 5 per cent each of fractions A and C. The results are shown in table 4.

Table 4 shows how an increasing concentration of fraction B of a casein acid digest shows an increasing inhibition of the growth of this streptococcus. Commercial preparations of proline and hydroxy-proline, when employed separately, or together with the basal medium, showed that these amino acids were not inhibitory to the growth of this organism. Since these substances are not responsible for the inhibition noted with the B fraction, it is probable that some substances are set free or formed during the fractionating or purification process.

THE EFFECT OF AMINO ACIDS ON GROWTH OF *Streptococcus rheumaticus*

A number of amino acids were added to the basal medium, separately, to determine their influence on the growth of this

TABLE 5

The influence of various amino acids on the growth of Streptococcus rheumaticus

AMINO ACID ADDED	GROWTH INDEX
Basal medium alone	1.00
A. Aliphatic, monoamino, monocarboxylic acids:	
Glycine	0.86
Alanine	1.20
Alpha-amino isobutyric acid	0.90
Valine	1.17
Norleucine	0.91
Leucine	0.90
Isoleucine	0.88
Serine	1.01
Alpha-amino hydroxy normal butyric acid	0.91
Cysteine	1.07
Methionine	1.09
B. Diamino, monocarboxylic acids:	
Arginine	1.54
Lysine	1.07
C. Monoamino dicarboxylic acids:	
Glutamic acid	1.20
Aspartic acid	0.90
Asparagine*	1.11
D. Aromatic amino acids:	
Phenyl alanine	1.07
Tyrosine	1.00
E. Heterocyclic amino acids:	
Tryptophane	0.83
Histidine	1.24

* Aspartic acid amide.

organism. In view of the fact that fraction C of the hydrolysate induced the best growth of this streptococcus, the amino acids which are known to be in this fraction, arginine, lysine, histidine, aspartic acid, glutamic acid and tryptophane were studied. In addition, certain monoamino, monocarboxylic acids including the sulfur-bearing acids cysteine and methionine were

added. Other substances believed by certain investigators to be important in the nutrition of animals were also included. While different concentrations of amino acids were studied the data presented in the table below are only for media containing the equivalent of 2 mgm. of amino acids per 10 cc. of culture media.[4]

An examination of table 5 shows that many of the amino acids studied when added to the basal medium favored the growth of *Streptococcus rheumaticus*. The greatest stimulation is apparent with the dibasic amino acid arginine which is present in the C fraction of the hydrolysate. Other amino acids of this fraction such as histidine, lysine and glutamic acid also stimulated the growth of this organism whereas aspartic acid and tryptophane appeared to be inhibitory.

In the monoamino, monocarboxylic group of acids, alanine, valine and to a lesser extent methionine and cysteine, were the only substances which stimulated the growth of this streptococcus. The remaining acids in this group including the leucine isomers, glycine and alpha-amino beta hydroxy N. butyric acid were apparently inhibitory.

Studies with combinations of amino acids showed little, if any, differences from the effect of adding amino acids separately.

DISCUSSION

In this study, dealing with the nitrogen metabolism of a non-hemolytic streptococcus, it is necessary to remember that the results are conditioned to a certain extent by the basal medium employed. The scant data available indicate in meat extract the presence of certain nitrogenous constituents including cystine. More recently Hughes (1932) and Knight (1935) found that beef extract contains a "growth factor" said to belong to the order of "natural bases." Knight (1937) showed that this growth factor could be completely replaced by synthetic preparations of nicotinic acid amide and vitamin B_{-1} (aneurin). Therefore, the results which may be observed, when one amino acid is added to a basal medium of this nature, must be interpreted with caution.

[4] The concentration of 2 mgm. per 10 cc. of medium was selected because less than this amount failed to stimulate growth and more than this gave less clear-cut results.

This study showing that arginine and histidine, diamino acids, and glutamic acid, a dicarboxylic acid, and in addition alanine and valine of the monoamino group, stimulated the growth of *Streptococcus rheumaticus* shows a close similarity to the previously reported results of Maver (1930), Fildes and Richardson (1935), Fildes (1935), Mueller (1935) and Sahyun *et al.* (1936) on the amino acid requirements of other organisms.

The inhibitory effect of tryptophane on the growth of this streptococcus is interesting in view of the ability of other bacteria to synthesize and use this amino acid, Fildes, Gladstone and Knight (1933), Sahyun *et al.* (1936).

It is hoped that studies now in progress using the chemically known growth factors present in beef extract in place of the complex extract itself will give further information regarding the nutrition of these streptococci.

SUMMARY

1. Employing a photoelectric colorimeter as a means of estimating the turbidity produced by the growth of *Streptococcus rheumaticus*, it was found that the monoamino, monocarboxylic acid fraction of a casein acid hydrolysate does not support growth; and that the proline fraction inhibits growth; while the butyl-alcohol insoluble fraction and the unfractionated hydrolysate stimulates growth.

2. The inhibitory action of the proline fraction, even in low concentrations, was shown to affect the growth of this streptococcus. Such inhibition was not caused by the amino acids, proline or hydroxyproline.

3. A study of the amino acids known to be present in the butyl-alcohol insoluble fraction showed that arginine, histidine, and glutamic acid, in the order named, stimulated the growth of this organism. Lysine and aspartic acid had little appreciable effect and tryptophane an inhibitory effect on its growth.

4. A few monoamino, monocarboxylic amino acids were studied. Alanine, valine, and to a lesser extent cysteine and methionine, stimulated the growth of this organism.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MARYLAND BRANCH

GOUCHER COLLEGE, BALTIMORE, FEBRUARY 23, 1939

THE ANTIBACTERIAL EFFECTS OF THE ORGANIC MERCURIAL COMPOUNDS. With special reference to their use as germicides for the sterilization of surgical and dental instruments. *John H. Brewer*, Hynson, Westcott & Dunning, Inc., Baltimore.

An improved centrifuge technique was described which was developed to determine whether the mercurials under optimal conditions are capable of destroying spores of anaerobes. The outstanding features of this technique are as follows: a sealed tube is used in which the organisms are exposed to the chemical agent for twenty-four hours, during which time the mercurial spore mixture is agitated constantly to prevent evaporation "rings" and to insure complete wetting of the entire inner surface of the tube. Settling or layering of the organisms is also prevented. Animal inoculation as well as cultural tests are used to determine the efficiency of the various mercurials with this technique.

It is to be concluded from the results of the tests described that none of the mercurials examined can be depended upon to sterilize instruments, when spore-forming organisms are present. In the case of *Clostridius tetani* none of the mercurials rendered these spores non-infectious after twenty-four hours' contact. The spores were still capable of infecting the mice and producing tetanus. The spores of the "clostridium septique" and *Bacillus anthracis*, however, could be rendered non-infectious in certain instances. That is, although it was possible to recover these organisms in cultural experiments, they did not produce an infection when injected into animals.

ROUTINE TESTING OF DISINFECTANTS AND THE SCIENTIFIC ATTITUDE. *C. M. Brewer*, Food and Drug Administration, D. C.

In routine biological tests, even those with as simple a technique as ordinary disinfectant and antiseptic methods, non-uniform and unexplained results are frequently encountered. Officials in the Food and Drug Administration have an unusual opportunity of observing the large number of workers experiencing difficulty in obtaining satisfactory results. It is impossible in the description of routine methods to point out the serious influences which minor and apparently innocuous deviations from prescribed technique may have. When technicians are entrusted with the responsibility of performing routine biological tests, they should be under the supervision of scientifically-minded and broadly trained persons: Examples were cited.

Too complete filtration of the subculture media, in one instance, pre-

vented proliferation of small numbers of test-organism; and illustrated the importance of recognizing the types of death-curves obtained in disinfecting testing as well as the phenomenon of allelocatalysis. In another case, the resistance of the test-organism was affected by reducing the diameter of the culture tubes by 5 mm., which reduced the resistance of the organism. This brought out the importance of the surface-area-volume ratio and the necessity of a proper accessibility of oxygen for obtaining resistance of the test-culture.

A recently proposed standard of technique for sporicidal methods to be used for evaluating chemical sterilizers of surgical instruments was cited as a flagrant example of unscientific methods.

HISTORICAL APPROACH TO THE STUDY OF ANTIBACTERIAL ACTION. *Justina Hill*, Johns Hopkins Hospital.

Man's empirical use of antibacterial agents far preceded his knowledge of micro-organisms. This is exemplified by primitive methods of preserving food, by Egyptian mummification, by the Hindu use of copper vessels for water and by some of the surgical techniques of the Hippocratic corpus. Even from the strange decoctions of herbal medicine true antiseptics may be recovered. We now use alcohol where the ancients used wine, and acetic acid where they used vinegar, but the active principles were early recognized. Clinically, establishment of the fact that gunpowder itself was not poisonous and Paré's accidental discovery that wounds did not have to be treated with boiling oil opened the long road towards a more rational therapy against local infection. The modern era of testing antibacterial drugs began with Sir John Pringle when in 1750 he presented the Royal Society his method for evaluating antiseptics. This included the use of a relatively standard medium, controlled temperature conditions and the establishment of a unit for comparison, sea salt. Within the bacteriological era it must not be forgotten that Lister was an experimental bacteriologist, constantly studying antiseptic substances in tests "conducted in the early morning hours while most men slept in bed." In this country we are proud to remember the work of Sternberg, which he started between Indian fights at Walla Walla in 1878. From Koch's 1881 paper to the present phenol coefficient tests progress has been direct. We now need in part (1) an accurate method for testing the bacteriocidal action of highly bacteriostatic drugs, such as the mercurials (2) a standard bacteriostatic method comparable in inoculum and in as many ways as possible with the bacteriocidal test, in order that bacteriocidal-bacteriostatic ratios may be determined, (3) more agreement in regard to amount of protein to be used, possibly whole blood for drugs intended for clinical use and (4) standard methods for testing the action of drugs in wounds and on the skin.